SPECIFIC GROWTH INHIBITORS OF *RALSTONIA SOLANACEARUM*

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DEDICATION

This work will not have been complete without the love and support of my lovely parents Mr. Ombiro, Mrs. Moige and siblings Bro. Cliff, Bro. Bismarck and Sister Cate. To the almighty Lord, I can only say thank you for the good health and endless blessings.

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ABSTRACT

Phytopathogenic bacteria are the leading causes of crop yield losses worldwide. Therefore, finding effective and safe compounds to control bacterial plant diseases is an ongoing effort in current plant pathology. One of the most destructive and therefore important phytopathogenic bacteria is *Ralstonia solanacearum* (*Rs*), a causal agent of bacterial wilt diseases in many solanaceous crops such as tomato, eggplant, pepper and so on. Control of this pathogen has been done through chemical control, soil fumigation, breeding for resistant cultivars, cultural control, use of plant-derived metabolites and bio-control. Despite the available control efforts, the pathogen continues to be a serious menace in solanaceous crop production, and there is no effective bactericide to control bacterial wilt diseases. Therefore, this study endeavored to identify newer chemical alternatives to enhance the control of the pathogen.

We screened the effective compounds that specifically inhibit growth of *Ralstonia solanacearum* from 376 chemical compounds in the pilot libraries of the antibiotics laboratory RIKEN Japan. As a result of screening, I identified one promising compound, 1-(4-bromophenyl)-6-methoxy-1,2,3,4-tetrahydrobeta-carboline that inhibited the growth of *R. solanacearum* (*Rs*1002), and designated it as ralhibitin A. Analysis of chemical structure and growth inhibitory activity revealed that halogens at the para position of the benzene ring was necessary for activity to inhibit the growth of *R. solanacearum*. Contrastively, presence of different group at the para position such as a methyl or a hydroxyl at the ortho or meta position abolished the activity of the compounds. The methoxy group of ralhibitin A was not necessary as an analog compound lacking it was still active. An analog compound having a benzene ring

instead of a methoxy group of ralhibitin A lost activity. Based on this characterization, I obtained functionally active five compounds, ralhibitin A to E, and assessed their activity to inhibit *Rs*1002 growth in detail. The halogen of ralhibitins at para position include; a bromine (ralhibitins A and C), a chlorine (ralhibitins B and D), and a iodine (ralhibitin E), with ralhibitins A and B possessing a methoxy group, but the others do not. Then, I revealed that all ralhibitins have a specific activity to inhibit growth of *Rs*1002 at least > 5 µg/ml final concentration. The most effective compounds are ralhibitin A, C and E that completely inhibited the growth of *Rs*1002 at 1.25 µg/ml. Ralhibitins (A, B, C and D) specifically inhibited the growth of *R. solanacearum* and *Xanthomonas oryzae* pv. *oryzae*, but they did not inhibit that of the other bacteria tested at 10 µg/ml final concentration. However, Ralhibitin E besides inhibiting *R. solanacearum* and *X. oryzae* pv. *oryzae* was able to completely inhibit the growth of *X. campestris* pv. *campestris* and Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* at 10 µg/ml. Growth inhibition activity of these compounds was stable at pH 6-9 and even after autoclaving.

In this study, I identified the compounds ralhibitins A-E as effective growth inhibitors of plant pathogenic bacteria *R. solanacearum*. Therefore, I investigated *in planta* activity of ralhibitin D, which is soluble in ethanol to protect tomato seedlings against *R. solanacearum.* Disease symptoms was scored as disease indexes, and the degree of disease suppression was evaluated as control efficiency, which was obtained by the formula 100(C-T)/C, in which C and T are disease indexes of control group and ralhibitin-treated group, respectively. As a result, ralhibitin D significantly suppressed tomato bacterial wilt in a concentration dependent manner. The control efficiency was 96.3 at 9 days post inoculation (DPI) with 111 µg/ml of ralhibitin D, and 87.7 at 10 DPI with 56 µg/ml of ralhibitin D under lower bacteria concentration (OD_{660nm} = 0.06). Disease suppression was also observed at higher bacterial concentration

(OD_{660nm} = 0.2). The control efficiency was 75.4 at 10 DPI with 111 μg/ml concentration of ralhibitin D, and 69.3 at 10 DPI with 56 μg/ml concentration.

Most bacteria are tolerant to ralhibitins, and only limited bacteria including *R. solanacearum* are sensitive to ralhibitins. However, the sensitivity mechanism of *R. solanacearum* to ralhibitins is still not clear yet. To determine the mechanism of how ralhibitins inhibit the growth of *R. solanacearum*, I investigated the effect of spent culture medium of insensitive bacteria with ralhibitins on the growth of *R. solanacearum* (*Rs*1002), and found that spent cultures did not inhibit the growth of *R. solanacearum*. This result indicated that the ralhibitin-insensitive bacteria may be able to inactivate the inhibitory effect of ralhibitins. Therefore, I conducted a transposon mutagenesis experiment that may identify the genes related to ralhibitin-tolerance using ralhibitin-insensitive bacterium *Pseudomonas syringae* pv. *tomato* DC3000. In this screening, the transposon miniTn5 was randomly inserted into the genome of *P. syringae* pv. *tomato* DC3000 by conjugation, and ralhibitin-sensitive transconjugants were selected. However, strong candidates for the genes involved in tolerance to ralhibitins were not obtained yet.

Based on these findings, ralhibitin D can be a potential lead compound for a bactericide to control bacterial wilt disease in tomato, hence decreasing yield losses and ensuring increased production of solanaceous crops such as tomato and eggplant.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Origin of *R. solanacearum*

Ralstonia solanacearum is a Gram-negative bacterium causing diseases in crops of the solanaceous family such as potato, tomato and pepper, and so on (Peeters et al., 2013).The first description of *R. solanacearum* occurred in 1908 by Smith (Li et al., 2014). The pathogen exists as a *Ralstonia solanacearum* species complex (RSSC) because of many strains that are different genetically (Prior et al., 2016). Infection by this pathogen occurs through the roots of a susceptible host, and pathogens move through the cortex, into the intercellular space and finally colonizes the xylem tissues (Bae et al., 2015). Reports have shown that there are over 67 sequenced *R. solanacearum* genomes (Liu et al., 2017). The *Ralstonia solanacearum* strains have different origins; GMI1000 (tomato) from French Guyana, ICMP7963 (Potato), UW551 (Geranium) from Kenya, UW491 (Potato) from Colombia, MolK2 (Banana) from Philippines, CFBP1416- (Plantain) from Costa Rica, CIP417 (Banana) from Philippines, UW163 (Plantain) from Peru, Po82 (potato) from Mexico, CFBP6783 (Heliconia) from French west Indies, K60 (Tomato) from United states, Grenada91 (Banana) from Grenada, IPO1609 (Potato) from Netherlands, JT516 (Potato) from Reunion Islands, CMR43 (Potato), CMR34 (Tomato) from Cameroon, AP31H, AP42H, TB1H, TB2H, TC1H (Potato) from Uruguay, CIP10 (Potato) from Peru, CIP418 (Peanut) from Indonesia, Ant75 (Heliconia), Ant307 JY200, Ant1121 (Anthurium) from Martinique, CFBP7014 (Anthurium) from Trinidad, B34 (Banana) from Brazil, A3909 (Heliconia) from Hawaii, NCPPB332 (Potato) from Zimbabwe, *R. syzygii* R28 (Cloves) from Indonesia, R288 (Morus alba) from China, PSS190 (Tomato) from Taiwan, MAFF301558 (Potato) from Japan, CFBP2047 (Tomato) from USA (Ailloud et al., 2015;Guidot et al., 2009: Cellier et al., 2017). Previously, *R. solanacearum* strains were classified into 4 phylotypes namely; phylotype 1, phylotype II, phylotype III and phylotype IV. However, the current classification groups the strains into 3 genospecies (*R. syzygii, R. solanacearum* and *R. pseudosolanacearum*) (Fegan and Prior, 2005; Prior et al., 2016; Safni et al., 2014)

1.2 Importance of *Ralstonia solanacearum*

With the increasing world population, the demand for food is also increasing requiring doubling of food production. The constraints of food production include; weeds, pests and pathogens, with diseases caused by pathogens responsible for 14% yield reduction (Agrios, G.N., 2005). The most limiting diseases include soil borne pathogens like *Ralstonia solanacearum* accounting for between 10-20% of yield losses per year, followed by airborne and seed borne diseases (USDA, 2003). Soil borne *R. solanacearum* is the one of the most important plant bacterial pathogen (Mansfield et al., 2012). Many countries consider *R. solanacearum* as a quarantine and bioterrorism pathogen (Cellier et al., 2017). The disease is very destructive and causes fast and fatal crop wilting subsequently resulting in yield losses. Level of damage is dependent on pathogen strain, type of soil, climatic conditions, pattern of cropping and host (Elphinstone, 2005). For instance, the disease cause between 80-100% losses in banana, 33-99% in potato, 0-91% in tomato, 20% in groundnut and around 10-30% in tobacco (Elphinstone, 2005).

1.3 *Ralstonia solanacearum* epidemiology

Many epidemiological surveys were first used to monitor human and animal diseases but have since been involved in plant disease identification. The surveillance includes collection of data, its analysis and subsequent interpretation providing key information on disease management. Diagnosis and identification of *R. solanacearum* is hampered by the existence of the pathogen as species complex (Cellier et al., 2017). One of the first methods for epidemiological surveillance is Multilocus sequence typing (MLST) and was first developed by clinical microbiologists involved with *Neisseria meningitides* (Maiden et al., 1998). Multilocus sequence analysis (MLSA) and MLST are commonly used in bacterial disease identification, distribution of bacteria strains and their phylogenetic relationships (Hanage et al., 2005; Maiden, 2006). This method uses nucleotide sequences of selected housekeeping genes to decipher the role of recombination and mutations on evolution and diversity of diseases (Urwin and Maiden, 2003; Feil et al., 2004). Amongst the pathogens that have utilized this method to

understand the phylogenetic relationships include; *Xanthomonas* spp. (Hamza et al., 2012) *Ralstonia solanacearum* species complex (RSSC) (Wicker et al., 2012) *Clavibacter* spp. (Jacques et al., 2012) and *Salmonella* spp. (Roumagnac et al., 2006). The distribution of *Ralstonia solanacearum* is wide but epidemics are common in the subtropics and tropics (Hayward, 1994). However, there are cold tolerant strains in the temperate regions (Elphinstone, 2005).

1.4 *Ralstonia solanacearum* morphology and structure

Ralstonia solanacearum strain (UY031) bacterium is Gram-negative, non-sporulating, aerobic and has rod-shaped cells of length 0.5 to 1.5 μm. Attenuation index (AI) has been used in determining the pathogenicity of *R. solanacearum* (Liu et al., 2004). This index refers to the ratio of red spot diameter to the whole colony diameter (Fig.1.1) (Zheng et al., 2014). The morphology of *R. solanacearum* colonies has been used to classify the pathogen into virulent, intermediate virulent and avirulent strains (Liu et al., 2004). Accordingly, virulent strains have an AI of <0.65, avirulent >0.75 and intermediate strains with an AI of 0.65-0.75. The colony characteristics of virulent strains include presence of pink colony at the center, irregular in shape, big white edge and greater mobility whereas the intermediate virulent colonies have dark red spotted center, less mobile, surface humidity, small white edge. The avirulent colony is round in shape, less mobile, small or no white edge and dark red spot at colony center (Fig. 1) (Zheng et al., 2014).

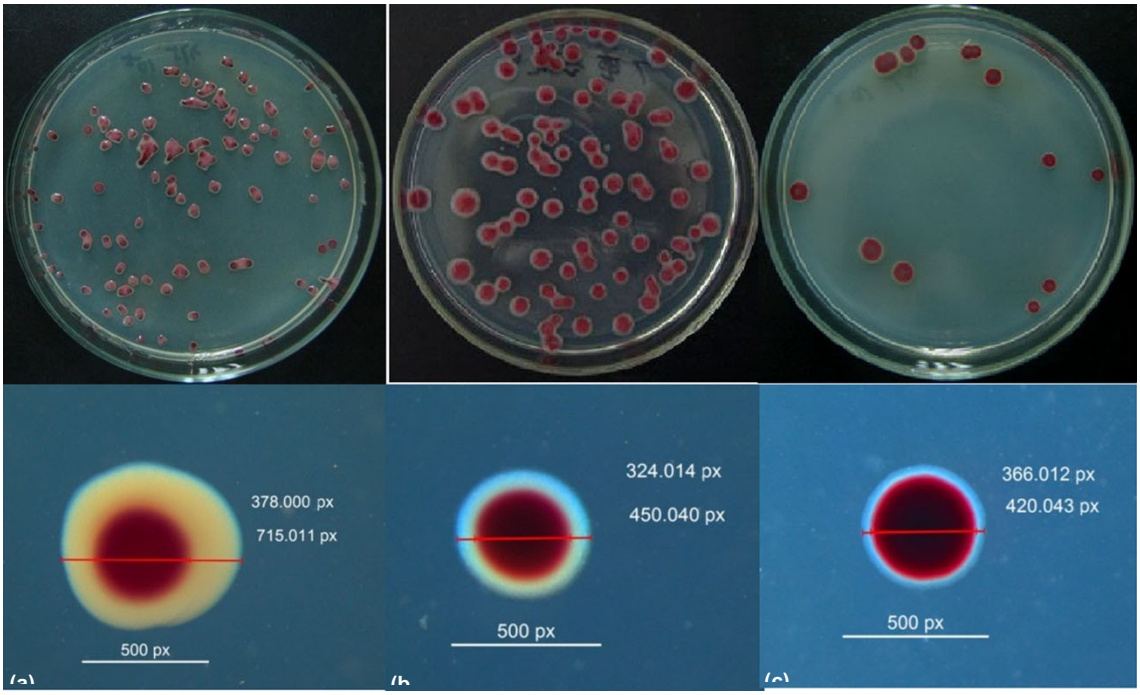

Fig. 1.1. Characteristics of *Ralstonia Solanacearum* strains a) virulent strains b) intermediate virulence c) avirulent strains (Zheng et al., 2014).

1.5 *Ralstonia solanacearum* Host Range

Ralstonia solanacearum infects over 250 plant species among then 54 dicot and monocot families (Elphistone, 2005). Some host specific strains have been reported such as brown rot of potato and Moko strains affecting banana (Peeters et al., 2013). Studies to identify host specific related genes have not been successful (Cellier et al., 2012; Guidot et al., 2007) Different interaction between some solanaceae crops (eggplant, tomato, pepper) and twelve *R. solanacearum* strains have been reported. The study on host pathogen interaction unraveled 6 phenotypes correlated with strain aggressiveness on the host (Lebeau et al., 2011). Being a flexible pathogen, *R. solanacearum* adapts easily to the environment and infects new hosts. A previously absent avirulent phylotype IIB-4NPB banana *R. solanacearum* strain from Martinique (Wicker et al., 2007) has been reported. The strains clustered with Moko pathogenic strains but don't cause disease in banana (Peeters et al., 2013). The strains have been reported to infect previous unrecognized *R. solanacearum* hosts such as ornamental plants, and cucurbits. They have also been found in weeds, water and wild species of solanaceae crops

(Wicker et al., 2007). There is lack of clarity on the cause of the emergency of the new strains but suggestions point to the vegetable/banana rotation programs practiced in Martinique. This is because the new strains were obtained from tomatoes/cucurbits planted on fields previously planted with banana (Wicker et al., 2009). The strains have also been reported in Brazil, a Moko prevalent region and hence it's postulated that the IIB-4NPBs originated from Brazil spreading to Martinique via infected ornamental anthuriums (Peeters et al., 2013).

1.6 *R. solanacearum* detection methods

Due to their complex nature, it is difficult to characterize *R. solanacearum* strains (Cellier et al., 2017). The species identification of *R. solanacearum* has been done by use of immune strips assays but their efficacy has been hampered by production of many false positives (Danks and Barker, 2000). This had led to the common use of DNA approaches that utilize PCR for species identification. Identification of *Ralstonia solanacearum* is through PCR using species specific primer pair 759/760 and phylotype specific primers (Nmult:23:AF, Nmult:21:2F, Nmult:21:1F, Nmult:22:RR, Nmult:22:InF) (She et al., 2017). The combination of these PCR methods with sequencing has enabled the development of phylogenetic trees (Cellier et al., 2017). The PCR evolution has resulted to newer and efficient techniques such as LAMP-PCR or qPCR (Lenarčič et al., 2014; Inoue and Nakaho, 2014). The diagnostic tools available for *R. solanacearum* detection have been compiled (Cellier et al., 2017) (Table 1.1).

Table 1.1. Primers used in the detection of *R. solanacearum* phylotypes

PCR names	Phylogenetic group	Primers name	Target	Primer sequence	bp	Bibliographic References
Phylotypes	I	Nmult:21:1F	ITS	CGTTGATGAGGCGCGCAATTT	144	Fegan and Prior, 2005
Multiplex	II	Nmult:21:2F		AAGTTATGGACGGTGGAAGTC	372	
	III	Nmult:23:AF		TTACSAGAGCAATCGAAAGATT	91	
	IV	Nmult:22:InF		ATTGCCAAGACGAGAGAAGTA	213	
	Na	Nmult:22:RR		TCGCTTGACCCTATAACGAGTA	na	
Moko	Seq 3	MUS35-F	Uncharacterized	GCAGTAAAGAAACCCG	401	Prior and Fegan,

Multiplex				GTGTT		2005a
		MUS35-R		TCTGGCGAAAGACGGGATGG		
	Seq 3	IS24-F	ISRso19	TCGGGCGTGAAGAGGCAGAC	490	Bagsic-Opulencia et al., 2006
		IS24-R		GGAGGTGTGCGCCATCAACTG		
	Seq 4	MUS20-F	*RhiG*	CGGGTGGCTGAGACGAATATC	351	Prior and Fegan, 2005a
		MUS20-R		GCCTTGTCCAGAATCCGAATG		
	Seq 4 PB	MUS06-F	Uncharacterized	GCTGGCATTGCTCCCGCTCAC	167	Prior and Fegan, 2005a
		MUS06-R		TCGCTTCCGCCAAGACGC		
	Seq 6	SI28-F	Uncharacterized	CGTTCTCCTTGTCAGCGATGG	220	Prior and Fegan, 2005a
		SI28-R		CCCGTGTGACCCCGATAGC		
	Seq 24	VC46-F	Uncharacterized	CTCCTGGGAGTCGGTTGGGTC	100	Woo et al., unpublished
		VC46-R		AGGGAACCTAGGCGTGACTG		
IpxC	*Rssc*	759	*IpxC*	GTCGCCGTCAACTCACTTTCC	282	Opina et al., 1997
		760		GTCGCCGTCAGCAATGCGGAATCG		
pehA	*Rssc*	pehA 3	*pehA*	CAGCAGAACCCGCGCCTGATCCAG	480	(Huang and Schell, 1990)
		pehA 6		ATCGGACTTGATGCGCAGGCCGTT		
pehB	*Rssc*	RS3	*pehB*	AGCACGACCGGTGCGACCTGCT	822	(Glick et al., 2002)
		RS4		CACCCCGCGCGTGTCGTCGTAG		
fliC	*Rssc*	fliC F	*fliC*	GAACGCCAACGGTGCGAACT	400	Schonfeld et al., 2003
		fliC R		GGCGGCCTTCAGGGAGGTC		
BP4	*Rssc*	BP4-R	Unknown—Cloned RAPD fragment	GACGACATCATTTCCACCGGGCG	1102	Lee and Wang, 2000
		BP4-L		GGGTGAGATCGATTGTCTCCTTG		
16S rRNA	*Rssc*	OLI1	16S	GGGGGTAGCTTGCTACCTGCC	288	(Seal et al., 1993)
		Y2		CCCACTGCTGCCTCCCGTAGGAGT		
mutS	*Rssc*	mutS-RsF.1570	*mutS*	ACAGCGCCTTGAGCCGGTACA	758	Prior and Fegan, 2005a
		mutS-RsR.1926		GCTGATCACCGGCCCGAACAT		
egl	*Rssc*	Endo-R	*Egl*	GCGTTGCCCGGCACGAACACC	800	(Fegan, M., and Prior, 2005)
		Endo-F		ATGCATGCCGCTGGTCGCCGC		
16S rRNA	*Rssc*	27F	*16S*	AGAGTTTGATMTGGCTCAG	48	(Taghavi et al., 1996)

		1492R		GGTTACCTTGTTACGACTT		
16S-23S rRNA ITS region	*Rssc*	1100F	ITS	GCAACGAGCGCAACCC	50	(Lane, 1991)
		240R		TTCGCTCGCCACTACT		
16S-23S rRNA ITS region	*Rssc*	L1	ITS	AGTCGTAACAAGGTAGCCG	48	Fegan et al., 1998
		PS-23Sr		TACTACGTCCTTCATCG		
Brown rot	Seq1 & Seq2	630	Genomic DNA fragment "prophage region"	ATACAGAATTCGACCGGCACG	307	Fegan et al., 1998
		631		AATCACATGCAATTCGCCTACG		
IIB-4NPB	IIB-4NPB	5F	Genomic DNA fragment	GCGCGCGAGGCTGGTGATGT	661	(Cellier et al., 2015)
		5R		TGGGTTCGCAGGCGGACAGC		
Moko	Moko & NPB	93F	*KfrA*	CGCTGCGCGGCCGTTTCAC	477	Cellier et al., 2015)
		93R		CGGTCGCGGCATGGGCTT		
BDB	BDB	121F	Uncharacterized	CGTATTGGATGCCGTAATGGA	344	Tan, 2003
		121R		AAGTTCATTGGTGCCGAATCA		
BDB	BDB	BDB2400-F	*GpS*	GCTGACTATAGGCACAGCGG	131	
		BDB2400-R		AATCGCCGTTCCCATACAAG		

1.7 Biodiversity of *Ralstonia solanacearum*

Characterization of *R. solanacearum* strain GMI1000 (Phylotype 1) in the early 21st century enabled greater molecular understanding of the versatility and pathogenicity of the organism (Li et al., 2016). On the basis of host range *R. solanacearum* has been classified into 5 races while classification based on metabolism of hexose alcohols and disaccharides yielded 6 biovars (Liu et al., 2017). Grouping of *R. solanacearum* into phylotypes was achieved through similarities of internal transcribed spacer region, *endoglucanase* gene and the hypersensitive reaction and pathogenicity gene (*hrp*) (Fegan and Prior, 2005). There are four phylotypes and 53 sequevars namely; Phylotype I (Asia), Phylotype II (America), Phylotype III (Africa) and Phylotype IV (Indonesia) (Fegan and Prior, 2005). Recent studies through DNA-DNA hybridizations have grouped *R. solanacearum* into 3 genospecies (*R. solanacearum*, *R. pseudosolanacearum* and *R. syzygii*) i. e. phylotype II composed of *R. solanacearum* strains, phylotype I and III made up of *R. pseudosolanacearum* strains and *R. syzygii* subsp. *syzygii* subsp. nov., having strains in phylotype IV including *R. syzygii* subsp. *celebesensis* subsp. nov., and *R. syzygii* subsp. *indonesiensis* subsp. nov., (Prior et al., 2016). Among the phylotypes, phylotype I has wide host range affecting both woody and herbaceous plants (Hayward, 1994).

The genome of *R. solanacearum* evolves through horizontal gene transfer and recombination (Wicker et al., 2012). Recombination was shown to be driven by phylotype IV via gene donation with phylotype I considered the most recombinogenic lineage while phylotype IIB was a clonal group (Peeters et al., 2013). Genomic islands which are regions in the genome of organisms with a region of about 5 kb containing coding sequences lacking synteny to other similar genomes. Genomic islands have been found in genomes of some *R. solanacearum* strains (CFBP2957, CMR15) and are made up of bacteriophages, insertion sequences (mobile elements) or putative type III effectors suggesting horizontal gene transfer (Remenant et al., 2010). In GMI1000 phylogenetic analysis has revealed 151 foreign genes (Fall et al., 2007). There has also been reports of between strains transfer of DNA fragments (30-90 kb) but only in the laboratory an indication of horizontal gene transfer (Coupat et al., 2008). This transfer implies that future strains could become highly virulent.

Diversity studies have shown that *R. solanacearum* exists as a species complex containing both virulent and avirulent strains. These strains differ in their pathogenicity, distribution and host range (Fig. 1.2) (Zheng et al., 2014) Previous *R. solanacearum* classification characterized host range (races) and biovars (carbohydrate utilization) (Hayward, 1964, 1991). Race 3 biovar 2 is among cool strains affecting potato is the only existing coherent race (Clarke et al., 2015). This potato pathogen is considered a quarantine pest in North

America and Europe (EPPO, 2015). It also affects the ornamental plant geranium with latently infected cuttings suspected to have caused the spread of the disease from Africa to Central America, North America and Europe (Williamson et al., 2002; Janse et al., 2004). However, due to the difficulty in distinguishing biovars and races, current classification using proteomic and genomic methods is based on phylotypes.

Fig. 1.2. Pathogenicity of different strains of *R. solanacearum* on its host tomato. a) Virulent strains b) intermediate virulence c) avirulent strains (Zheng et al., 2014).

1.8 Distribution of *R. solanacearum*

Occurrence of bacterial wilt has been mapped to both the tropical and temperate regions of the world (Hayward, 1991). Greatest economic losses have been reported in Brazil, USA, Colombia, Indonesia and South Africa (Wicker et al., 2007). *R. solanacearum* has wide geographical locations with reports of 31% incidence in Bangladesh affecting eggplant (Hussain et al., 2005). Other reports show incidence of 25% in potatoes and 55% in chili in Ethiopia, 15% losses of tomatoes, 10% loss in aubergine and capsicum in Philippines (Bekele et al., 2011;Zehr, 1969). Phylotypes I and IV have been identified in Japan (Horita et al., 2014 ; Waki et al., 2013), phylotypes I, II and IV identified in India, (Sagar et al., 2014), phylotypes 1, II and III identified in Africa (N'Guessan et al., 2012) and phylotype I and II identified in China (Xu et al., 2009)

1.9 Infection and virulence factors for *R. solanacearum*

The entry of the pathogen *R. solanacearum* in the plant occurs through roots moving onto the cortex, intercellular spaces and finally colonizes the xylem vessels and vascular system (Fig. 1.3) (Hikichi et al., 2017). Secretion of exopolysaccharides into the xylem vessels results to the wilting of plants (Bae et al., 2015). The pathogen can overwinter in the soil for long periods of time providing material for future infection (Bae et al., 2015). Virulence in this pathogen is

enhanced by the presence of the type III secretion system (T3SS) (Coll and Valls, 2013). This system injects effector proteins into the cytosol facilitating infection (Erhardt et al., 2010; Tampakaki et al., 2010). There has been a report of about 72 *Ralstonia* injected proteins (Rips) in *R. solanacearum* RS1000 (Mukaihara et al., 2010). These effector proteins are delivered into the plant cells by the T3SS inhibiting or modulating the plant defense system such as PAMP-triggered immunity (PTI) (Boller and He, 2010). However, recognition of the effector proteins by host derived R protein may lead to hypersensitive reaction (HR) due to the effector-triggered immunity (ETI). This mechanism also known as programmed cell death enhances host resistance to pathogens through pathogen movement restriction from infection site (Oh et al., 2010)

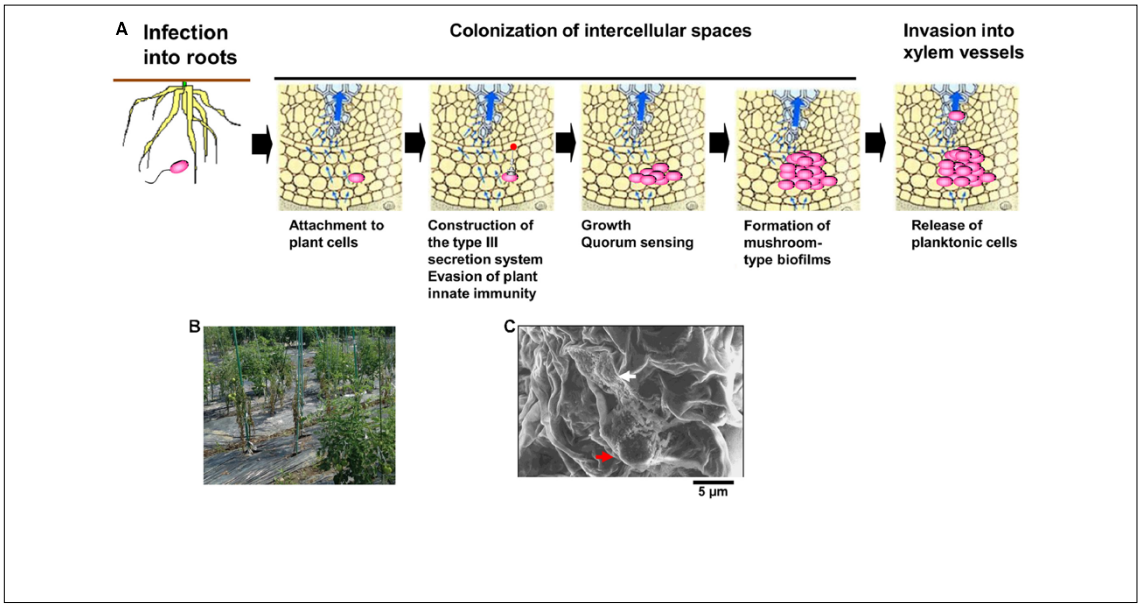

Fig. 1.3. A, *R. solanacearum* infection strategy within the intercellular space, B, Wilting symptoms caused by *R. solanacearum*, C, biofilm structures (Mushroom type) of *R. solanacearum* in infected tomato (Hikichi et al., 2017).

2.0 Control of *R. solanacearum*

The ability of bacterial wilt to overwinter in the soil and water for many years in the absence of the host makes it a challenging pathogen to control (Champoiseau et al., 2009) Some of the common control measures include inoculum elimination, the use of resistant varieties and rootstocks together with prevention of the disease through frequent epidemiological surveillance (Yahiaoui et al., 2017). The major control methods include; chemicals, biological control, the use of plant derived compounds, soil disinfection and breeding for resistance (Mao et al., 2014;Yuliar et al., 2015)).

2.0.1 Chemical control

Triazole derivatives have got a diversity of bioactivities against bacteria, viruses and fungi (Küçükgüzel et al, 2001; Li et al., 2016). These are key nitrogen-containing compounds that have potency against bacteria (Fan et al., 2018). They also have fungicidal activities as illustrated by important commercial agro fungicides like flusilazole, triadmenol and triadimefon. The heterocycle 1, 2,4-triazole ring has been used in creating new functional molecules (Küçükgüzel and Çikla-Süzgün, 2015). This is because it can bind to different enzymes and receptors through non-covalent interactions leading to greater biological potency (Kulabaş et al., 2016). Commercial products with the 1,2,4-triazole ring include letrozole (anti-cancer), ribavirin (viricide), fluconazole and triamiphos (fungicides). Novel triazole derivatives containing quinazoline moiety were shown to have signify antibacterial activity against pathogen *R. solanacearum*. The minimum inhibitory concentration (MIC) against *R. solanacearum* was at 81.6 µg/ml (Fan et al., 2018).

The quinazoline scaffold is considered a significant moiety that produces molecules with significant biological activities such as antimalarial, anti-cancer and anti-tumor, insecticidal and antimicrobial potency (Zhang et al., 2018). There are many current agrochemicals and drugs with quinazoline moiety in the markets such as fenazaquin (acaricide), gefitinib (anticancer) and prazosin (high blood pressure drug) (Fan et al., 2018). There is a growing interest for quinazolinone compounds from both agricultural and medical chemists due to their perceived antiviral, antibacterial and antitumor activities. Fungicides cloroqualone is the compounds containing quinazolinone moiety. Some quinazolin derivatives have been shown to have antibacterial activity to both Gram-negative and Gram-positive pathogens with MIC of 0.8-3.3 mg/ml (Shi et al., 2013). Another recent study also showed antibacterial activity of some quinazolin derivatives against *R. solanacearum* (Zhang et al., 2018).

Indoles

Some 1H-indol-2-ol derivatives have been shown to have inhibitory effects on *R. solanacearum* (Tu et al., 2018). They showed a MIC of 10.25 µg/ml against *R. solanacearum* (Tu et al., 2018). These results showed that these compounds are better than existing bismerthiazol compounds used to control the pathogens. In these compounds, antibacterial activity was improved by the addition of a halogen group to the benzene ring. However, the use of electron withdrawing group on the benzene ring decreased the activity remarkably implying that electron-withdrawing groups enhance the activity of compounds. The indole

group contains nitrogenous heterocycle with many reports confirming the critical role played by the indole moiety in biological potency (Cacchi et al., 2005; Takada et al., 1984). For example, alkaloids with indole moiety have shown broad-spectrum activity i. e. antitumor and anticancer activities (Bao et al., 2005; Kouko et al., 2005)

Sulfone derivatives have a wide range of activities as herbicidal, antifungal and anticancer agents (Shi et al., 2015); Xu et al., 2012). A sulfone-attached heterocycle has been involved in the activity of this sulfone derivatives (Xu et al., 2012). Commercial compounds with this moiety include cyazofamid, tolyfluanid and amisulbrom (Xu et al., 2012). There have been reports of inhibitory effects of some sulfone derivatives that can control *R. solanacearum* (Milling et al., 2011). Much lower MIC has been reported for sulfone derivatives (2-(methyl/ethyl sulfonyl)-1,3,4-oxadiazole derivatives) against *R. solanacearum* (0.45 µg/ml) (Su et al., 2017). Other sulfone derivatives containing 1,3,4-oxadiazole moieties have also been reported to have inhibitory effect against *R. solanacearum* with MIC of 32.1 µg/ml. The effect was much greater than a commercially marketed bactericide saisentong, copper formulation (Xu et al., 2012).

Coumarins

Some plant derived compounds such as coumarins have also been reported to inhibit the growth of *R. solanacearum* (Yang et al., 2016). These compounds contain fused beta pyrone and benzene rings. They have demonstrated anticancer, anti-coagulant, antibacterial and anti-oxidant properties (Barot et al., 2015; Céspedes et al., 2006). In Gram-negative bacteria, they have been shown to damage the cell membrane thereby effecting antibacterial activity (Yang et al., 2016). Other natural coumarin compounds with inhibitory activity against *R. solanacearum* include resveratrol and protocatechuic acid. Amongst coumarins with activity against *R. solanacearum*, daphnetin had the greatest activity with MIC of 64 µg/ml (Yang et al., 2016).

2.0.2 Biological control of *R. solanacearum*

There has been an increased interest in the research and use of biological control agents (BCAs) in disease management due to the environmental effects of the use of chemicals (Whipps, 2001). The advantages of BCAs include self-propagation after initial application, environmental friendliness, extended disease control (Quimby et al., 2002;Whipps et al, 2007). Their activity is through antibiosis, parasitism, induction of resistance, degradation of cell wall by enzymes and nutrient competition with pathogens (Agrios, 2005; Cook and Baker, 1983). The control

of bacterial wilt has been dominated by avirulent strains of bacteria such as *Pseudomonas* spp., *Bacillus* spp., *R. solanacearum* spp., and *Streptomyces* spp. (Yuliar et al., 2015). Other species that have been found to control *R. solanacearum* include; *Clostridiaum* spp. (Momma, 2008)), *Ralstonia picketii* (Wei et al., 2013), bacteriophages (Álvarez et al., 2007; (Yamada et al., 2007) Enterobacter (Xue et al., 2009) *Paenibacillus marcerans* (Li et al, 2011) and *Bacillus thuringiensis* (Zhou et al., 2008). Direct inoculation of *B. thuringiensis* to plants for protection against *R. solanacearum* induced production of defense genes like *PR-1* leading to resistance (Takahashi et al., 2014). *Bacillus amyloliquefaciens* SQR-7 has been reported to control bacterial wilt in tobacco increasing yields by 38%. The mode of action was shown to be the production of siderophores and indole acetic acid (Yuan et al., 2014). Similarly, a 73% control efficiency was observed when *Ralstonia picketii* QL-A6 was used to control bacterial wilt in tomato with mode of action shown to be competition for nutrients (Wei et al., 2013). Some fungal pathogens like *Glomus versifyirme* have been shown to control bacterial wilt in tomato with decrease of bacterial counts in the xylem tissues by 81.7 % (Zhu and Yao, 2004). The application methods include, seed coating, drenching or root dipping with drenching reported to have had lower efficacy (Yuliar et al., 2015). Efficacy of BCAs is hampered by a number of factors such as, poor and inconsistent colonization, challenges of mass inoculum production, efficacy in narrow host range (Whipps et al., 2001).

2.0.3 Plant derived compounds

There are reports of bioactive compounds against *R. solanacearum* from plants due to essential oils, metabolites and volatile oils (Vu et al., 2017a). Existence of this compounds have increased the interest on bio pesticides from plants (Bhagat, 2014). Plant extracts and secondary metabolites have potential utilization in the development of new bio pesticides (Vu et al., 2017). However, statistics from commercially developed pesticides indicate that only <0.1% pesticide products are from plants (Sola et al., 2014; Vu et al., 2017). Some of the bio pesticides used currently include neem, essential oils and pyrethrum (Vu et al., 2017). Extracts from allium have been shown to decrease the infection by *R. solanacearum* on tomato plants after soil application (Deberdt et al., 2012). *Eichhorina crassipes* and *Lantana camara* from Ethiopia have also been reported to possess antibacterial activity against bacterial wilt in tomato (Alemu et al., 2013). Gallotannins from *Sedum takesimense* were reported to have antibacterial activity against *R. solanacearum* with 1,2,3,6-tetra-O-galloyl-β-glucose being the most active (Vu et al., 2013). Some plant metabolites such as thymol, lemongrass oil and palmarosa oil were reported to also significantly reduce the severity of bacterial wilt

(Pradhanang et al., 2003; Vu et al., 2017). Clove oil isolated from *Eugenia caryophyllata* has been shown to be bioactive against bacterial wilt. There was a significant reduction in bacterial wilt incidence when treated with clove oil (Lee et al., 2012). Another type of oil from Cinnamon was shown to have inhibitory activities against *R. solanacearum* complex. Development of disease symptoms was reduced 3 days post inoculation after treatment with 0.01% cinnamon oil (Lee et al., 2012). An extract from *Toxicodendron sylvestre* known as methyl gallate has shown antibacterial activity against *R. solanacearum* with a reported MIC of 20 µg/ml. Efficacy in the greenhouse has been capped at 62.5% better than streptomycin sulfate (Yuan et al., 2012). Methyl gallate was found to have inhibitory effect on *R. solanacearum* through suppressing extracellular enzymes such as pectinases, causing damage to its cell wall and interference to its energy metabolism by inhibiting respiration (Fan et al., 2014).

2.0.4 Breeding fore resistance

A key strategy in the management of bacterial wilt is breeding crops with wide spectrum resistance to the many strains existing (Salgon et al., 2017). In model plant species like *Arabidopsis thaliana*, broad studies on resistance mechanisms has been done leading to the identification of resistance genes *RRS1* interacting with an effector PopP2 (Codes for TIR-NBS-LRR protein) (Salgon et al., 2017). For resistance to *R. solanacearum* (GMI1000), the resistance gene *RRS1* has been reported to interact with Cys protease (Bernoux et al., 2008). The interaction of RRS1 with a *Pseudomonas syringae* resistance gene *RRS4* has greatly enhanced resistance to *R. solanacearum* strains (Sohn et al., 2014). The resistance to bacterial wilt in resistant tomato cultivar Hawaii 7996 is influenced by two minor and major quantitative loci (Salgon et al., 2017). The QTLs bwr-6, 12 have been reported to enhance resistance partially to phylotype I and IIB (Wang et al., 2000, 2013; Carmeille et al., 2006). Reports have shown that wildtype eggplant *Solanum torvum* has great tolerance to *R. solanacearum* and is widely utilized as rootstock in tomato and eggplant production to enhance their tolerance to the pathogen (Nahar et al., 2014). In eggplant variety *S. torvum* Sw. cv. Torubamubiga a rapid cell death (HR) was induced when *R. solanacearum* RS1002 was infiltrated into its leaves (Nahar et al., 2014). Screening of RS1000 effectors identified Rip36 to be the effector that facilitated the rapid cell death in *S. torvum* Sw. cv. Torubamubiga.

2.1 Development of antibacterial agents

The origin of antibacterial compounds is either from natural products, semisynthetic antibacterial or fully synthetic antibacterial agents. The discovery of penicillin from *Penicillium chrysogenum* as an antibacterial agent marked a great scientific breakthrough. Semi-synthesis occurs through chemical modification of a natural product as was previously done on tetracyclines and aminoglycoside. Despite the potential effects of natural compounds as active ingredients, their use is limited due to their low concentration and cost of exploitation (Szczepanik et al., 2016). Understanding of their chemical structures can drive discovery of more potent derivatives or analogues (Szczepanik et al., 2016). Modifications of β-damascone pharmacophore have led to analogs with greater antifeedant potency. Halolactones have been shown to exhibit greater anti-feeding activity against the pest *Alphitobius diaperinus* (Gliszczyńska et al., 2014). The bromo lactone was the most active at lower doses as compared to the chloro lactone against *A. diaperinus*. Synthetic compounds have been reported to have enhanced stability as illustrated by streptomycin hydrogenation to form dihydrostreptomycin with improved chemical stability. Similarly, chlorotetracycline obtained from *Streptomyces aureofaciens* was reported to have potency against both Gram-negative and Gram-positive bacteria. Cleaving of the carbon-chlorine bond through hydrogenolysis resulted in semi-synthetic tetracycline. This shows that natural products form the basis for antimicrobial discovery process (Wright et al., 2014). Antibiotic development has always been two-fold driven i. e. from modifying existing antibiotics to improve bioactivity or screens from the soil environment for new antimicrobial compounds.

2.2 Importance of halogens

Halogen elements include chlorine, fluorine, iodine and bromine. The halogen bond is also referred to as X-bond (Fanfrlík et al., 2015). The non-covalent X-bond is critical in drug interactions, crystal engineering and molecular recognition (Metrangolo and Resnati, 2001). These elements have 7 electrons and are considered oxidants. Halogens have been used as active ligands in the pharmaceutical industry (Jiang et al., 2016). Reports also have shown that most patents have compounds synthesized through the addition of halogen elements. Halogen bonds are orthologous to the hydrogen bond hence can be utilized in improvement of ligand affinities while maintaining structural interactions of compounds. This makes them easy targets for drug and inhibitors designs. Drug compounds have been improved through addition of

halogen groups to their structure (Shalas et al., 2018). Chlorine addition improved the analgesic potency of compounds hence increasing pain inhibition potential. The reason for this enhanced activity has been suggested to be that halogen elements slow down metabolic processes on the compound through obstruction. There have been reports of the use of bromine for water disinfection against viruses, protozoa and bacteria (Kim, 2014). Other reports indicate reduction in bacterial numbers after treatment with bromine at pH range of 7.0-7.8 (Goodenough, 1964).

Bromine at 4 mg/L was observed to decrease *E. coli* numbers after 10 minutes at 0 °C and pH 7.0 (Krusé et al., 1970). When compared to chlorine analog, it was determined that N-bromo oxazolidinone had fifty times more activity against *Staphylococcus aureus* (Williams et al., 1988). Comparatively, against *Entamoeba histolytica* parasite, it was found out that bromine had greater efficacy as compared to other halogen elements iodine or chlorine (Stringer et al., 1975). The efficacy of bromine against poliovirus was reported to be higher at both pH 7 and 9 when compared to chlorine where there was a decrease in its activity at pH 9 (Sollo et al., 1975). This implies that bromine provides more protection at wider pH ranges as compared to chlorine. The effect of halogens groups on antibacterial activity has been reported on sulfone derivatives against *R. solanacearum* (Su et al., 2017).

2.3 Chemical analogues

Analogues are important in the drug discovery process. Chemical structural modification has been reported to improve the biological activities (Shalas et al., 2018). The modification can either be done through substitution or aromatic ring homologation. For instance, adding a halogen to an aromatic ring of 4-chlorobenzoylthiourea has been reported to increase activity against pain (Shalas et al., 2018). The properties of a pharmacophore have been altered by changing the substituent position. This is because of the effect on distribution of electrons on the aromatic ring. There are many reports of increased activity of analogues as compared to the parent compound. Analogues of a peptide obtained from Oliver flounder fish (*Paralichthys olivaceus*) were reported to have enhanced activity against yeast and bacterial species (Nam et al., 2014). The analogues were synthesized through amino acid substitution, amidation or addition. For example, Gly residues were replaced by Lys or Trp residues in order to improve amphipathicity. Replacement with Lys residues was to enable determination of the role of cations on antibacterial activity (Nam et al., 2014). A compound in garlic named allicin has been reported to have broad activity against both Gram-negative and Gram-positive bacteria (Leontiev et al., 2018). The active functional group was reported to be thiosulfinate hence

analogue compounds were synthesized namely; dibenzyl-, dipropyl-, dimethyl-, diethyl-, and diallyl-thiosulfinates and assayed against *Saccharomyces cerevisiae* and bacteria. They were reported to have activity against bacteria species; *Micrococcus luteus*, *Pseudomonas syringae* pv. *phaseolicola* 4612, *Escherichia coli* K12, *Pseudomonas fluorescens* pf-01 and fungus *Saccharomyces cerevisiae* yeast strain BY4742 in addition to being thermally stable than the parent compound allicin (Leontiev et al., 2018).

Similarly, the use of fluorine increasing biological potency and stability has been reported (Shah and Westwell, 2007). Fluorine substitution on a potent compound profoundly enhances its pharmacological activity due to the high stability of its C-F bond in comparison with the C-H bond, its smaller size, the high electron withdrawing properties and high liphophilicity. High electronegativity of compounds has been linked to alteration of pharmacological properties of molecules. Another property of fluorine is the ability to decrease alkalinity of molecules making them more bioavailable (Shah and Westwell, 2007). Fluorine also enhances the affinity of molecule to bind to the target through direct interaction or increasing the polarity of other functional groups to interact with the target protein. The C-F bond is metabolically stable hence reducing metabolic attacks that influence metabolism of a drug.

A compound that inhibits the absorption of cholesterol SCH 48461 (Fig. 1.4a) has susceptibility to metabolic attacks through oxidation, hydrolysis and dimethylation. Fluorine substitution greatly improved the stability and enhanced in vivo efficacy leading to the development of the analogue drug Ezetimibe (SCH 58235) (Rosenblum et al., 1998). The new analogue had over 50 times more potency than the parent drug illustrating the potential of structure activity relationship. Other studies have also reported that substitution of fluorine with hydrogen reduced the toxicity of methotrexate by the development of gamma-fluoromethotrexate via increasing the acidity of the carboxyl functional group nearby (Fig. 1.4b) (Kokuryo et al., 1997). The modification of a thiourea pharmacophore through adding an allyl functional group and a benzoyl substitution had a positive effect on the activity of resultant analogues against pain (Shalas et al., 2018). The control of soil based pathogens and nematodes has been dependent on the use of methyl bromide (MeBr) (Ibekwe et al., 2010). The fumigant has a broad potency against many pathogens, which has made it a great success. However, there have been reports on its effect to the ozone layer leading to its phase out. Its analogue, methyl iodide synthesized by the replacement of bromine by iodine has reported more efficacies when compared to the parent compound (Ohr, 1996). This exemplifies the fact that

development of analogues through functional group substitutions can enhance further biological potency of compounds.

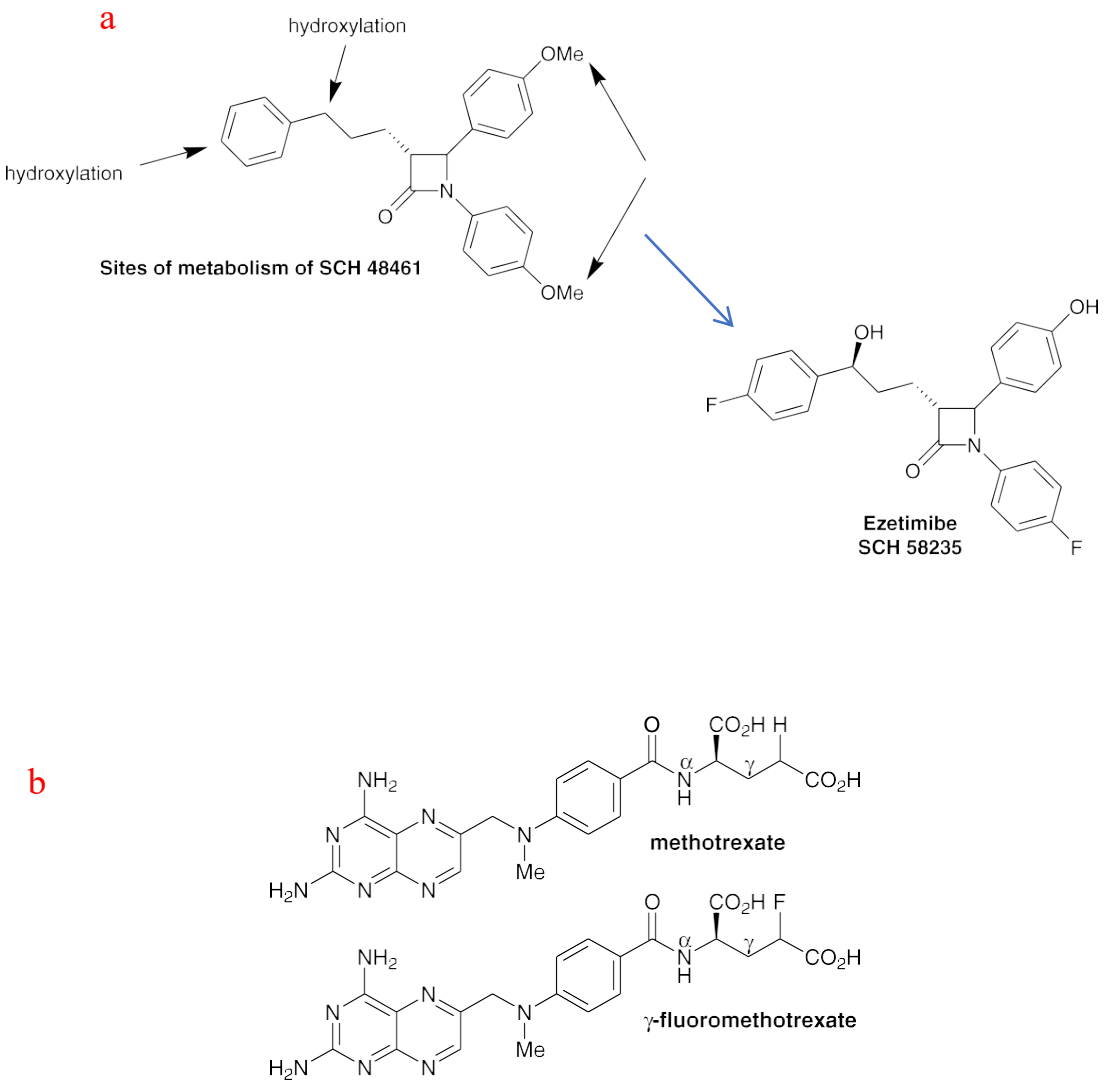

Fig. 1.4. a) Synthesis of a new effective compound Ezetimibe from SCH 48461. b) Reduction of toxicity of methotrexate through the synthesis of analogue gamma fluoromethotrexate.

2.4 Transposon mutagenesis

Bacterial diversity, speciation and evolution have been facilitated by lateral gene transfer (LGT) (McLellan, 2012). This transfer implies the non-generational genetic material movement. LGT occurs through either; conjugation which involves the movement of 'F' plasmids through direct contact in the bacterial cells, transduction where foreign DNA is introduced into bacterial genome by site-specific recombination and finally by natural transformation (Thomas and Nielson, 2005). Transposon insert into the host bacteria with bias to specific sequences, genomic locations and DNA with a specific structure (McLellan, 2012). The transposons; Tn7, Tn10 and Tn5 are greatly involved in rearrangement of genomes through insertions. Amongst the transposons, it has been reported that Tn5 is the most effective in mutagenesis of bacteria (Chaudhry et al., 2015). From this study, it has been found that Tn5 produces non-leaky, single insertion site mutations. Insertion of Tn5 to a pathogenicity related gene leads to loss of function of the gene. The resultant mutants may be important as biological control tools for the particular pathogen (Couteaudier, 1992).

Transposon mutagenesis for instance has been employed in determining the genes involved in tolerance to antibiotics in *Escherichia coli* (Hu and Coates, 2005). In *R. solanacearum*, transposon mutagenesis has been done using Tn5 system on isolates obtained from solanaceaous plants (Titarenko et al., 1997; Lin et al., 2008). Recently, transposon mutagenesis has been attempted on *R. solanacearum* isolates from a new medicinal host known as patchouli (*Pogostemon cablin*) (Blanco) Benth. In order to identify mutants with reduced virulence (Wang et al., 2019). In this system mutagenesis was done through electroporation by using *R. solanacearum* strain PRS-84 from Patchouli herb and transconjugants were selected for kanamycin resistance. Successful intergration of transposon in the genome of *R. solanacearum* was achieved and stable single site insertion mutants obtained (Wang et al., 2019). This system was able to obtain mutants with reduced virulence.

2.5 Aim of this project

The aim of this study was to screen and identify compounds that can control *R. solanacaerum*

The key objectives of this study are:

i) To screen and identify compounds that can inhibit the growth of *R. solanacearum*

ii) To identify the active functional group of effective compounds through structure activity relationship (SAR)

iii) To determine the activity of effective compounds against *R. solanacearum*

iv) To investigate the properties of effective compounds

v) To investigate the effect of *R. solanacearum* inhibiting compounds on other phytopathogenic bacteria

vi) To determine the in vivo protective activity of effective compounds against *R. solanacearum* in infected tomato seedlings

vii) To identify the target site for the effective compounds in bacteria through transposon mutagenesis

CHAPTER II

SPECIFIC GROWTH INHIBITORS OF *RALSTONIA SOLANACEARUM*

Geofrey Sing'ombe Ombiro, Taku Sawai, Yoshiteru Noutoshi, Yuta Nishina, Hidenori Matsui, Mikihiro Yamamoto, Kazuhiro Toyoda, Yuki Ichinose (2018). Specific growth inhibitors of *Ralstonia solanacearum, Xanthomonas oryzae* pv. *oryzae, X. campestris* pv. *campestris, and Clavibacter michiganensis* subsp. *michiganensis*. Microbiological Research 215 (2018) 29–35. https://doi.org/10.1016/j.micres.2018.06.005

I. SUMMARY

To control the pathogen *R. solanacearum*, chemical control and integrated pest management have been attempted. In this study, we sought new compounds that specifically inhibit the growth of *R. solanacearum*. As a result, we identified one promising compound, 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline, which inhibited the growth of *R. solanacearum* (*Rs*1002) from a pilot library of 376 chemicals provided from RIKEN. We further obtained its structural analogues and assessed their ability to inhibit *Rs*1002 growth. Then we identified five compounds, named ralhibitins A to E, that specifically inhibit growth of *Rs*1002 at >5 μg/ml final concentration. The most effective compounds, ralhibitins A, C, and E completely inhibited the growth of *Rs*1002 at 1.25 μg/ml. Ralhibitins effective against *R. solanacearum* were evaluated against other Gram-positive and Gram-negative bacteria pathogens using broth macro dilution method at a final concentration of 10 μg/ml. Ralhibitin A-E inhibited the growth of *R. solanacearum* strains and *Xanthomonas oryzae* pv. *oryzae* strains. Ralhibitin E containing an iodine group besides inhibiting *R. solanacearum* and *X. oryzae* pv. *oryzae* also completely inhibited the growth of *Clavibacter michiganensis* subsp. *michiganensis* and *X. campestris* pv. *campestris* at 10 μg/ml final concentration. Dose dependent assays revealed that the most effective ralhibitin against *X. oryzae* pv. *oryzae* was D at minimum inhibitory concentration (MIC) of 5 μg/ml while A, C and E had a MIC of 10 μg/ml. Ralhibitin B however had the least MIC at 15 μg/ml against *X. oryzae* pv. *oryzae*. On the other hand, the MIC of ralhibitin E against *C. michiganensis* subsp. *michiganensis* and *X. campestris* pv. *campestris* was 10 μg/ml. It has been reported that physical factors like pH and temperature affect antimicrobial activity in vitro. Similarly, storage conditions of antimicrobials can decrease their activity. The effect of different factors like temperature and pH on activity of ralhibitins was investigated. The results revealed that ralhibitins are stable at all the temperatures tested. Similarly, they were also stable at different pH levels. However, pH stability was affected at relatively lower pH of 6. Compounds that control bacteria work by killing effect or inhibition of bacterial growth. Ralhibitins showed a killing effect that was time dependent showing a significant reduction of *R. solanacearum* numbers at 24 and 48 hours post incubation.

II. INTRODUCTION

Exploitation of bioactive compounds is historical, dating back to Chinese, Egyptian, Arabic and Grecian civilizations, with the term ‘drug’ reported to have an Arabic origin (Lage et al., 2018). Bioactive compounds are chemical tools of defense against living organisms in

environments that are competitive. The previously used traditional bioactive discovery methods were halted due to; challenges in isolation of compounds, repeated re-identification of similar compounds and difficulties in using natural extracts in high throughput screening strategies (Lage et al., 2018). These methods have been substituted with synthetic libraries to screen for active compounds. There are two methods utilized in hit compound identification namely; target-directed drug discovery (TDD) and phenotype based drug discovery (PDD) (Lee et al., 2012). Under TDD, genes coding for proteins that have a role in causing a disease are identified and effective compounds that bind the target are obtained through screening (Lee et al., 2012). On the other hand, PDD entails a search for compounds causing alteration in a phenotype and their mechanism subsequently identified later on (Lee et al., 2012). There are different methods used for antimicrobial screening among which include; agar disk diffusion technique commonly utilized in susceptibility tests for antimicrobial compounds (Heatley, 1944) dilution method which is divided into agar and broth dilution methods respectively (Pfaller et al., 2004), time kill test mostly utilized for determining concentration and time dependent antimicrobial activity against pathogens (Klepser et al., 1998), poisoned food method for determining the antifungal effect of compound through measurement of growth inhibition diameter (Li et al., 2016), the use of thin-layer chromatography (TLC)-bioautography utilized in determining bioactivity of natural extracts (Horváth et al., 2010).

In *R. solanacearum*, there has been use of both TDD and PDD methods to identify effective compounds. For instance, screening of plant derived compounds against *R. solanacearum* by PDD identified effective compounds namely; coumarin and tea polyphenols possessing high antibacterial activity (Yang et al., 2016). Further, 18 coumarin derivatives were investigated for antibacterial activity against *R. solanacearum* and determined that they could inhibit the growth of the pathogen using the broth dilution method (Yang et al., 2016). Significant strong inhibitory effects were determined for the coumarins with a hydroxyl at the C-8, C-7 or C-6 positions. The most effective amongst the hydroxylated coumarins was daphnetin with MIC of 64 mg/L (Yang et al., 2016). There has also been a report of TDD in screening for compounds targeting virulence factors in *R. solanacearum* (Yang et al., 2017). Coumarins and its derivatives were screened to determine their effects on *R. solanacearum* type III secretion system (Yang et al., 2017). This study determined that umbelliferone, a hydroxylated coumarin was able to suppress expression of 6 type III effector genes namely; *ripD, ripX, ripR, ripW, ripTAL* and *ripP1* (Yang et al., 2017). The above results suggest the importance of multifaceted strategies to identify compounds that can suppress the plant pathogen *R. solanacearum.*

III. RESULTS

1. Identification of effective compound against *R. solanacearum*

In this study, I sought to identify compounds that can specifically inhibit the growth of *R. solanacearum* through screening of chemical libraries. The PDD approach was utilized in this study to identify compounds that can alter the phenotype of *R. solanacearum*. I screened a pilot library that was made up of 376 chemical compounds provided by antibiotics laboratory, RIKEN Japan by broth dilution method and identified one promising compound, named compound 1, 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline (Fig 2-1), which completely inhibited the growth of *R. solanacearum* (*Rs*1002) at a final concentration of 10 µg/ml. The pathogen *R. solanacearum* (*Rs*1002) is a spontaneous nalixidic acid resistant derivative from (*Rs*1000) (Mukaihara et al., 2004). Some antibiotics like novobiocin, rifampicin, spiramycin I, neomycin B, streptomycin, tetracycline hydrochloride, bicyclomycin were used as a positive control and they significantly inhibited the growth of (*Rs*1002) (data not shown) and I could confirm that the assay system was established. The promising compound had activity similar to that of the tested antibiotics confirming its antibacterial efficacy against *R. solanacearum.*

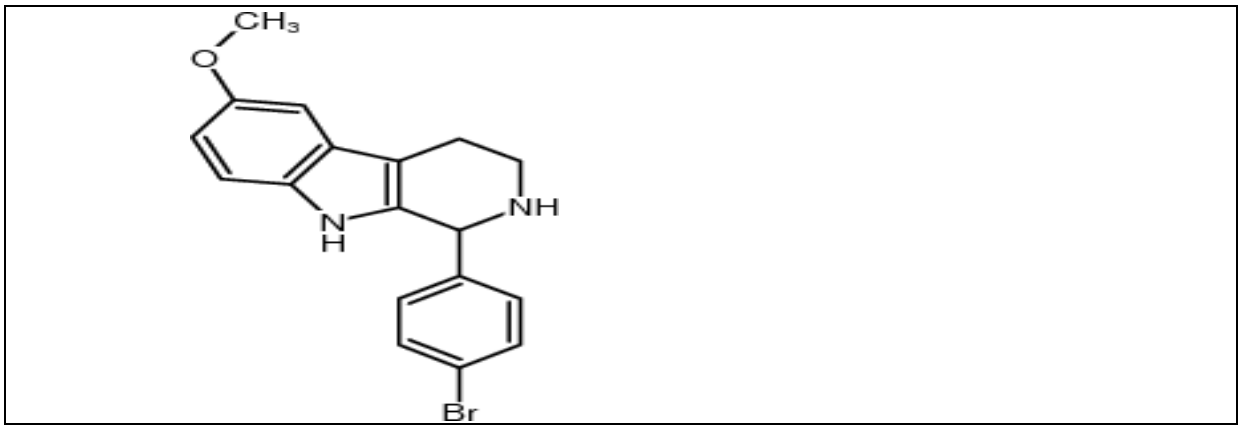

Fig 2-1. Structure of effective compound 1 **(1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline**) screened from chemical library.

2. Effective functional group

Determination of an active functional group in a structure can help in synthesis of analogues with similar activity or even better activity against pathogens. This can be done through structure activity relationship. I identified compound 1 that had activity to completely inhibit the growth of *R. solanacearum* at a final concentration of 10 µg/ml. To examine the relationship between chemical structure of compound 1 and inhibitory effect, I further analyzed the inhibitory effect of compound 1 and 8 other compounds with related structure. As shown in Fig. 2-2, among 9 structurally similar analogues including number 1, only number 1, 2 and

7 showed strong inhibitory effect on the growth of *Rs*1002. However, other similar compounds, number 3, 4, 5, 6, 8 and 9 are not effective to inhibit the growth of *Rs*1002 at a final concentration of 10 μg/ml. Therefore, the common structure in compounds, 1, 2 and 7, namely 1-phenyl-2,3,4,9-tetrahydro-1H-β-carboline, seems to be not enough but indispensable for the activity to inhibit the growth of *Rs*1002.

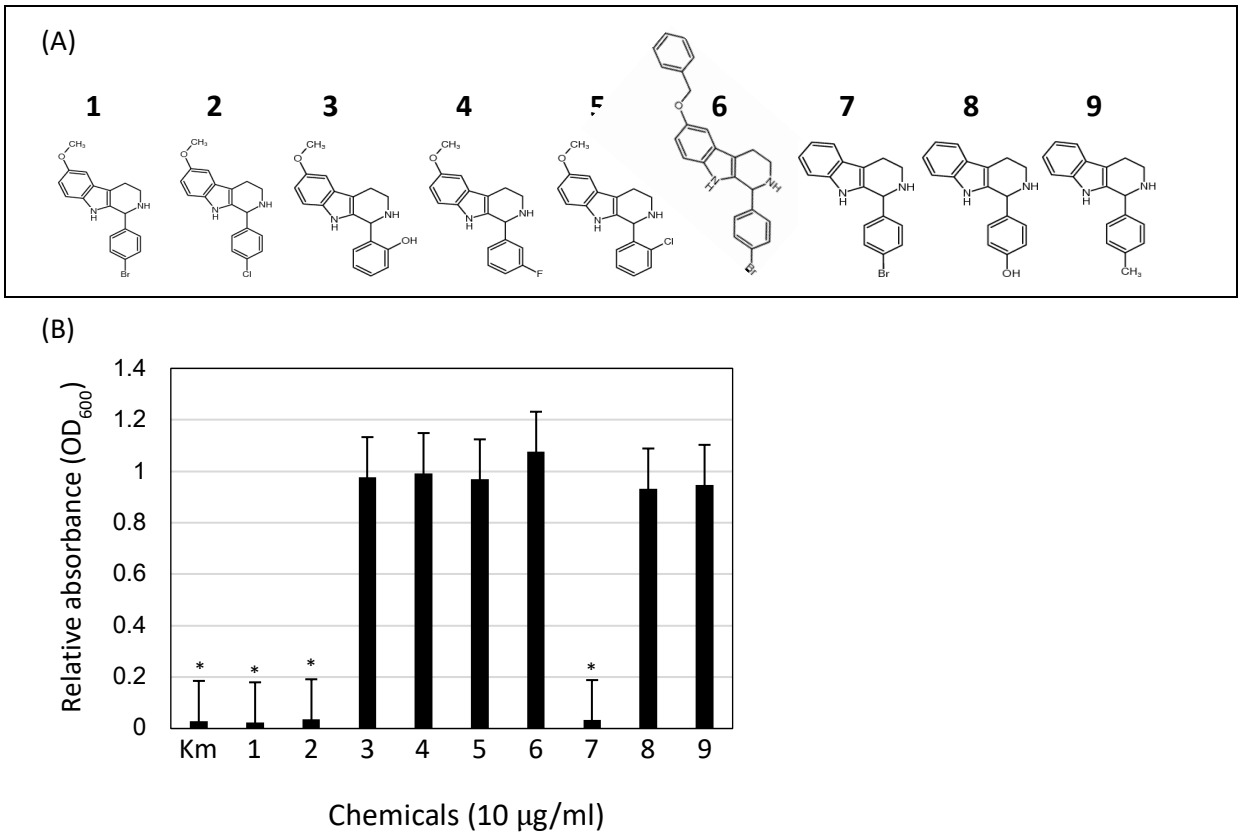

Fig. 2-2. Inhibitory effect of chemicals on growth of *R. solanacearum Rs*1002. (A) Structure of chemical compounds. Number 1 was screened from the pilot library, and numbers 2–9 are its analogues. (B) Activities of each compound to inhibit the growth of *Rs*1002. Overnight-cultured *Rs*1002 in 3 ml of BG medium was centrifuged, then bacterial density was adjusted to OD_{660} = 0.1 with fresh BG medium. One hundred and fifty μl of *Rs*1002 suspension was added to each well of a 96-well microtiter plate. As a control treatment 0.5 μl of DMSO was added, and 0.5 μl of each compound (3 mg/ml, DMSO) was added to obtain a final concentration of 10 μg/ml. After incubation for 24 h at 27°C in microtiter plates, the absorbance at OD_{600} was measured. The relative absorbance (OD_{600}) of the DMSO control was set to '1'. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations, and asterisks indicate that results are statistically significant compared to the DMSO control. (*P<0.001). The final concentration of kanamycin (Km) was 50 μg/ml.

3. Synthesis and activity of analogues

Structure activity relationship revealed that 3 compounds had activity to completely inhibit the growth of *R. solanacearum* at a final concentration of 10 µg/ml. The compounds include; 1, (1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline hydrochloride), 2 (1-(4-chlorophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline hydrochloride) from Pharmeks (Moscow, Russia) and 7 (1-(4-bromophenyl)-2,3,4,9-tetrahydro-1H-β-carboline hydrochloride) from Alinda Chemicals (Moscow, Russia), respectively. The compounds were renamed as ralhibitin A (compound 1), ralhibitin B (compound 2) and ralhibitin C (compound 7). A closer look at the effective compounds showed that they had a halogen (bromine or chlorine) at the para position of the benzene ring. Besides, effective compounds 1 and 2 had a methoxy group in their structure whereas compound 7 lacked a methoxy group confirming the non-essentiality of the methoxy group. To further proof this concept, I successfully synthesized 3 other analog compounds containing other halogens (chlorine, iodine and fluorine) and lacking a methoxy group in their structure namely; ralhibitin D, (1-(4-chlorophenyl)-2,3,4,9-tetrahydro-1H-β-carboline hydrochloride) ralhibitin E, (1-(4-iodophenyl)-2,3,4,9-tetrahydro-1H-β-carboline hydrochloride) and ralhibitin F, (1-(4-fluorophenyl)-2,3,4,9-tetrahydro-1H-β-carboline hydrochloride) (Fig. 2-3).

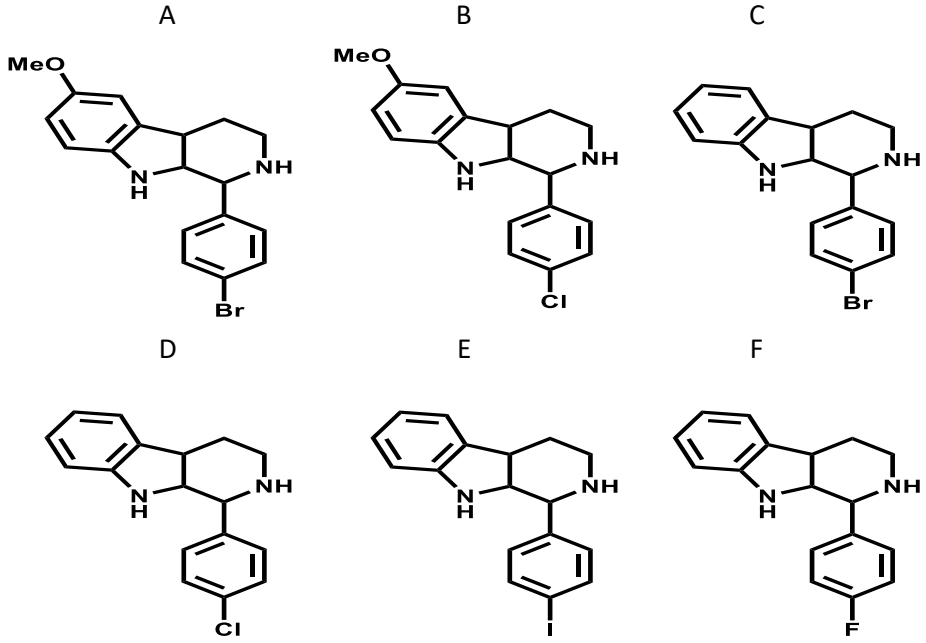

Fig. 2-3. Structures of ralhibitin A and its analogues ralhibitins B-F

4. Dose dependent activity of ralhibitins against *R. solanacearum*

I determined that ralhibitin A and its analogues (ralhibitin B and C) inhibit the growth of *R. solanacearum* at a final concentration of 10 µg/ml. Applications of compounds would require knowledge of specific dosages. Comparisons of the MIC between the original effective ralhibitin A and five other analogues can determine the most effective compound against *R. solanacearum*. I further varied the concentrations of ralhibitins A-F as from 10, 5, 2.5, 1.25, 0.625 and 0.31 µg/ml, respectively against *R. solanacearum*. Dose dependent activity revealed that ralhibitin A, C and E were the most active against *R. solanacearum* with MIC of 1.25 µg/ml. Ralhibitin B and D had MIC of 2.5 and 5 µg/ml, respectively while ralhibitin F had poor activity (Fig. 2-4).

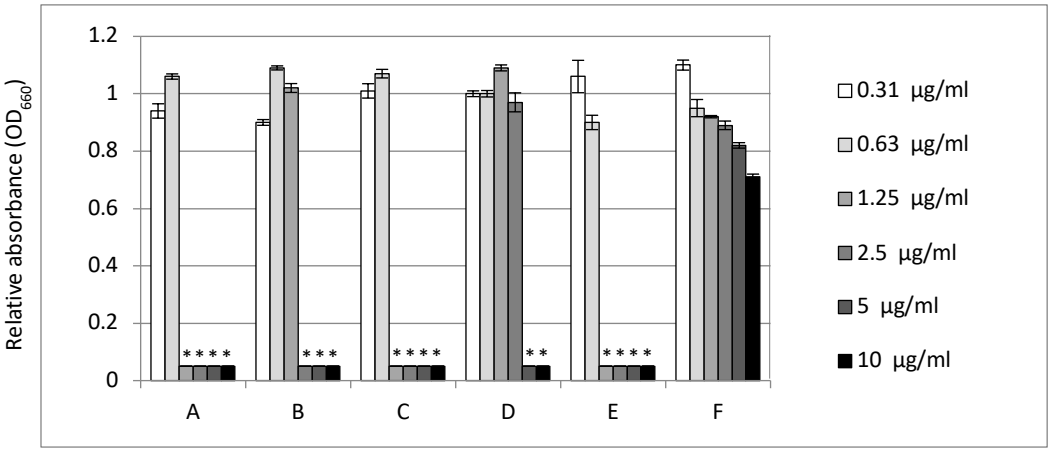

Fig. 2-4. Dose dependent activity of ralhibitins (A-F) against *Rs*1002. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at **P*<0.05 were considered significant.

5. Effect of temperature on activity of ralhibitins

To maintain activity, antibiotics storage is done at a range of temperatures. For instance, the recommended storage temperature for cephalexin is between (2-8 °C) and that of ciprofloxacin is (2-25 °C) (Sigma-Aldrich, 2003; Coleiro, 2012). Exposure to higher temperatures has been suggested to cause degradation, which would influence their effect on bacterial infections (Eisenhart and Disso, 2012). Therefore, it's important to investigate the stability of compounds to different conditions to determine the optimal storage ranges. To understand the temperature

stability of ralhibitins, they were subjected to varying temperatures and then their activity against *R. solanacearum* was determined. The results of thermo stability assay showed that the ralhibitins were stable after exposure to different temperatures (Fig. 2-5). Exposure of ralhibitins to very high temperatures, including autoclaving or very low temperatures did not affect their activity against *Rs*1002. This confirms the stability of the compounds qualifying them as potential control agents against *R. solanacearum* in natural condition that exposes compounds to different stress conditions.

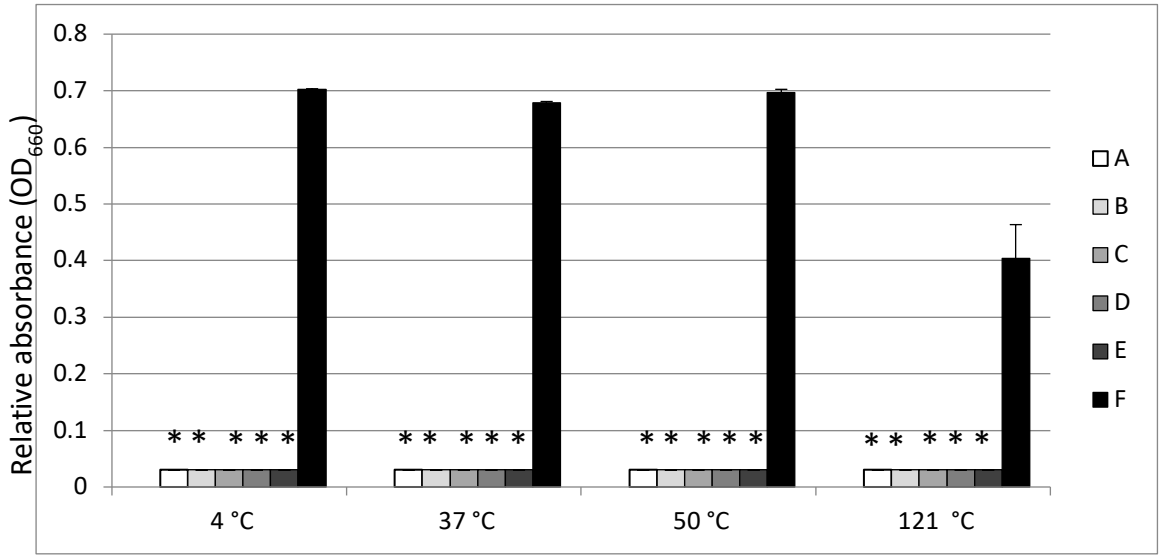

Fig. 2-5. Stability of ralhibitins. (a) Effect of temperature on inhibition of the growth of *Rs*1002 by ralhibitins. Ralhibitins were applied at a final concentration of 10 µg/ml. After incubation for 24 h at 27°C in a glass tube, the absorbance at OD_{660} was measured. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at $*P<0.05$ were considered significant.

6. Effect of pH on stability of ralhibitins

A study on bacterial growth inhibition by zinc nanoparticles showed that their activity was greatly influenced by medium pH. The study indicated that inhibition was increased by the change of pH to acidic (Saliani et al., 2015). The reason for this increased potency was attributed to nanoparticles dissolution that was enhanced at acidic conditions. However, the change from neutral pH 7 to alkaline pH did not have significant antibacterial activity against the tested bacteria (Saliani et al., 2015). *R. solanacearum Rs*1002 can grow in BG medium in a pH range of 6–9. An assay was done to determine the effect of pH changes in the medium on the activity of ralhibitins (Fig. 2-6). The results indicated that at pH 7 and 9, ralhibitins A to E

completely inhibited growth of *Rs*1002 at 10 µg/ ml. Although, growth inhibition by the ralhibitins (A-D) was slightly reduced at pH 6, that of ralhibitin E was not affected at pH 6. These results indicate that pH plays a role in the efficacy of some ralhibitins.

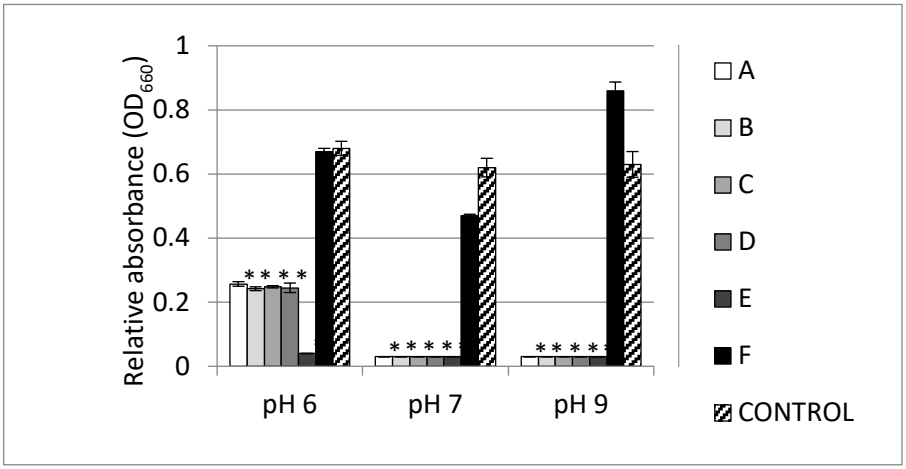

Fig. 2-6. Activity of ralhibitins on inhibition of the growth of *Rs*1002 at different pH of BG medium. Ralhibitins were applied at a final concentration of 10 µg/ml. After incubation for 24 h at 27°C in a glass tube, the absorbance at OD_{660} was measured. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at $*P<0.05$ were considered significant.

7. Killing activity of ralhibitins

To understand the pharmacodynamics of ralhibitins against *R. solanacearum*, a killing or inhibition assay was done. The assay monitored the activity of ralhibitin E over a time period through enumeration of bacteria populations at each time point and subsequent calculation of CFU/ml. The results indicated time dependent activity of ralhibitin E against *R. solanacearum* at a final concentration of 10 µg/ml. Bacteria numbers gradually decreased at 12 hours post incubation with a significant dipping of numbers occurring after 24 hours (Fig. 2-7).

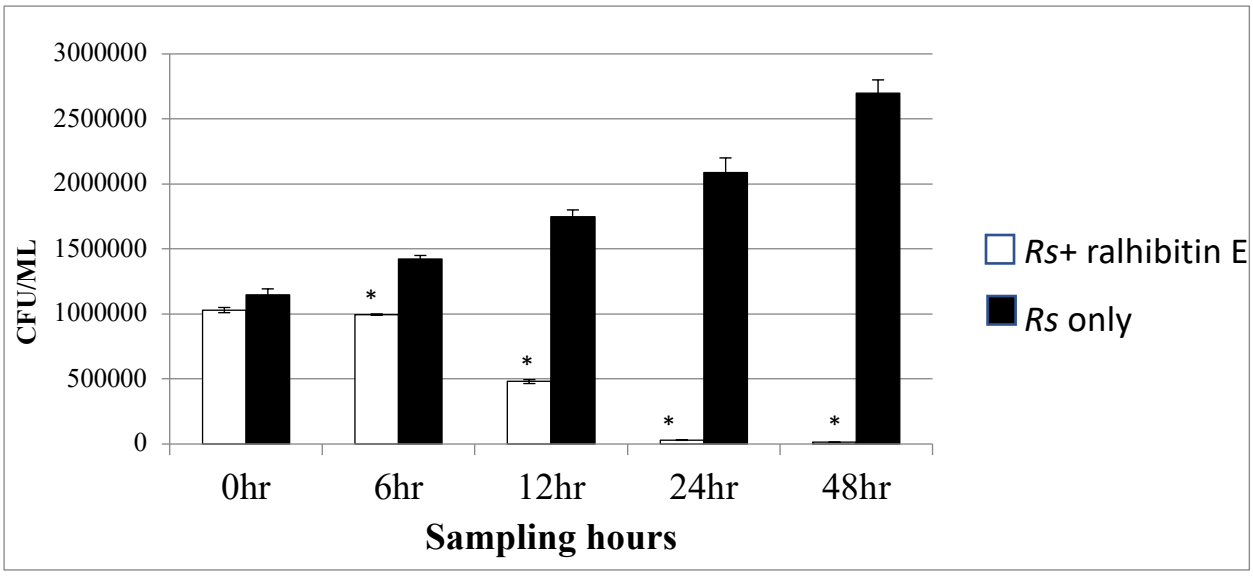

Fig. 2-7. Killing effect of ralhibitin E against *R. solanacearum (Rs).* The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at **P*<0.05 were considered significant.

8. Species specific activity of ralhibitins against phytopathogenic bacteria

Plant pathogenic bacteria have been classified in terms of their economic and scientific significance (Mansfield et al., 2012). The top 10 most important plant pathogenic bacteria include *Rhizobium radiobacter, Pseudomonas syringae* pathovar*s, X. oryzae* pv. *oryzae, Xylella fastidiosa*, *Erwinia amylovora, R. solanacearum*, *Xanthomonas axonopodis* pv. *manihots, Dickeya dadantii* and *Pectobacterium carotovorum* subsp. *carotovorum, X. campestris* pv. *campestris* (Mansfield et al., 2012). To identify chemical compounds that inhibit phytopathogenic bacteria is a critical goal in production of crops. My previous study identified compounds named ralhibitins A-E that could inhibit the growth of *R. solanacearum* RS1002 at a final concentration of 10 µg/ml final concentration and one compound, ralhibitin F that had poor activity. Investigation on the effects of these ralhibitins A-F on other plant pathogenic bacteria revealed interesting results. Ralhibitin (A-E) were able to inhibit the growth of four other *R. solanacearum* strains found in phylotype I and IV (Fig. 2-8). Similarly, ralhibitins A-E was able to inhibit the growth of *X. oryzae* pv. *oryzae* (*Xoo*). Strains of *Xoo* were also inhibited showing the broad range activity of these compounds. Ralhibitin E, besides inhibiting growth of *R. solanacearum* and *Xoo* also inhibited the growth of Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* and Gram-negative pathogen *Xanthomonas campestris* pv. *campestris* (*Xcca*) at 10 ug/ml final concentration (Fig. 2-8).

These results indicate that the synthesis of analogues from a parent compound (ralhibitin A) can expand the scope of activity as shown by the efficacy of ralhibitin E on other bacteria species both Gram negative (*Xcca, Xoo*) and Gram-positive (*Cmm*). Further, these results confirm proof of concept that the identification of active functional group in effective compound through structure activity analysis can guide the synthesis of new analogues, which can provide similar or even better potency against similar tested or other pathogens.

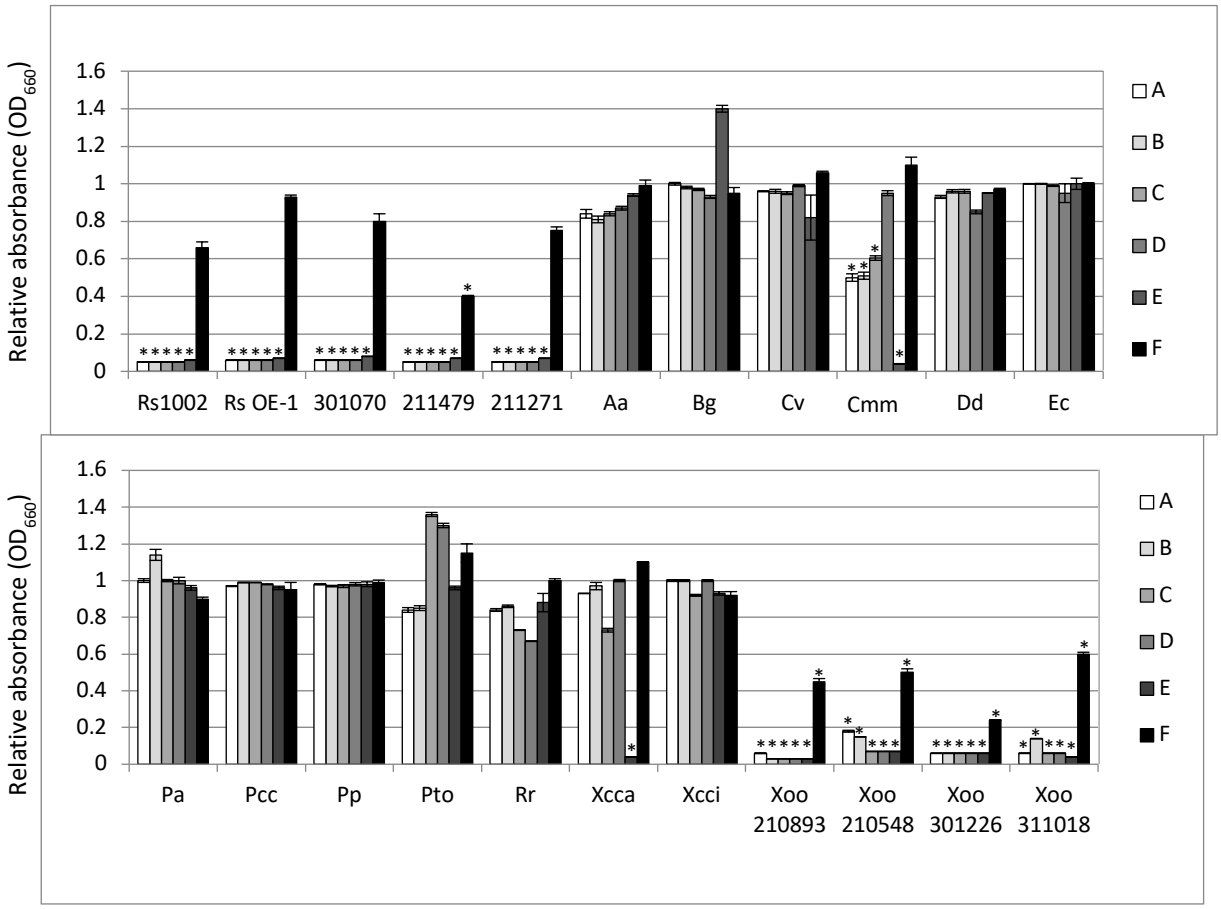

Fig. 2-8. The growth inhibition effect of ralhibitins on various bacterial strains. Various bacterial strains were incubated in 3 ml of medium with or without ralhibitins at 10 µg/ml for 24–48 h at 27°C in a glass tube, then the absorbance at OD_{660} was measured. The relative absorbance (OD_{660}) of the DMSO control was set to '1'. Results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at $*P<0.05$ were considered significant.

9. Dose dependent activity of ralhibitins against *Xanthomonas oryzae* pv. *oryzae*

An investigation into the effect of ralhibitin A-F on phytopathogenic bacteria determined that they could completely inhibit the growth of *X. oryzae* pv. *oryzae* (*Xoo*) strain 311018 at a final concentration of 10 µg/ml. To provide information on the specific dosage of the effective ralhibitins against *Xoo*, further investigation to determine the MIC was done. In this assay, a panel of ralhibitins A-E concentrations (15, 10, 5, 2.5, 1.25, 0.625 and 0.31 µg/ml) were used against *Xoo.* The results showed that ralhibitin D was the most active with a MIC of 5 µg/ml. The other compounds A, C and E had a MIC of 10 µg/ml, whereas ralhibitin B had MIC of 15 µg/ml. Ralhibitin F had poor activity even at highest concentration of 15 µg/ml (Fig. 2-9). The results show that analogue ralhibitin D is more effective than parent ralhibitin A supporting the hypothesis that synthesis of analogues can produce compounds with similar or better activity than the original compound.

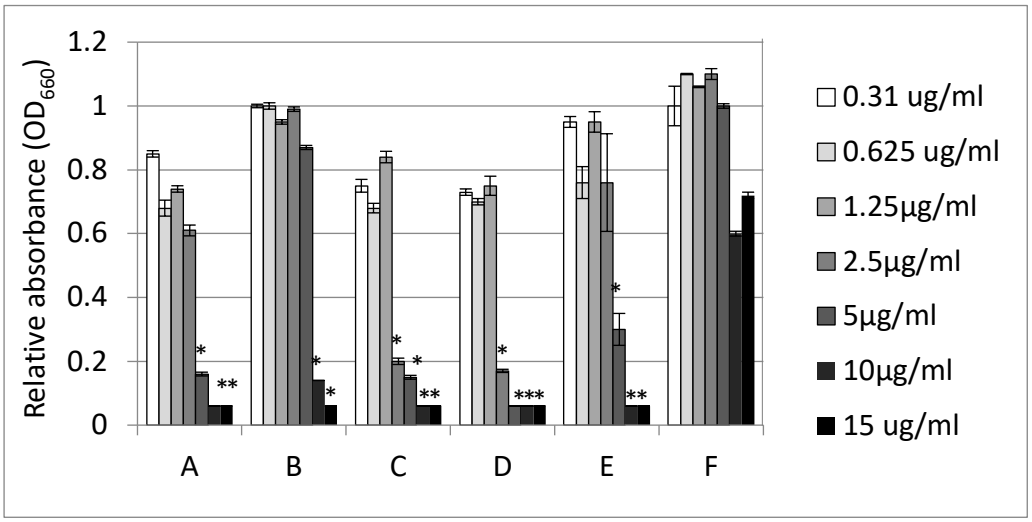

Fig. 2-9. Dose dependent of activity of ralhibitins against *X. oryzae* pv. *oryzae*. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at **P*<0.05 were considered significant.

10. Dose dependent activity of ralhibitin E against *X. campestris* pv. *campestris*

Black rot in cabbage is caused by *X. campestris* pv. *campestris* (*Xcca*) and is globally distributed (CABI, 2017a). The pathogen is Gram-negative and found in phylum proteobacteria (Rafael et al., 2018). It causes black rot disease in brassicas family (Lee et al., 2015). The use

of certified planting materials, breeding for resistance, insect vector control and destruction of plant debris have been frequently used in the management of *Xcca* (Rafael et al., 2018). The use of conventional pesticide has not been satisfactory due to low efficacy and negative effects to the environment. An investigation of the effect of ralhibitins A-F on different pathogenic and non-pathogenic bacteria revealed that ralhibitin E has a significant inhibitory effect against *Xcca* at 10 µg/ml final concentration. To further characterize the inhibitory activity, determination of MIC of this compound against *Xcca* was done. In this assay, ralhibitin E concentrations (10, 5, 2.5, 1.25, 0.625 and 0.31 µg/ml) were used against *Xcca*. Dose dependent results of ralhibitin E against *Xcca* showed that the MIC against *Xcca* was 10 µg/ml (Fig. 2-10). Inhibition of pathogens at a concentration of 10 µg/ml or below has been reported to be favorable for the development of new and effective control agents. This therefore means that ralhibitin E is a potential future bactericide against *Xcca*.

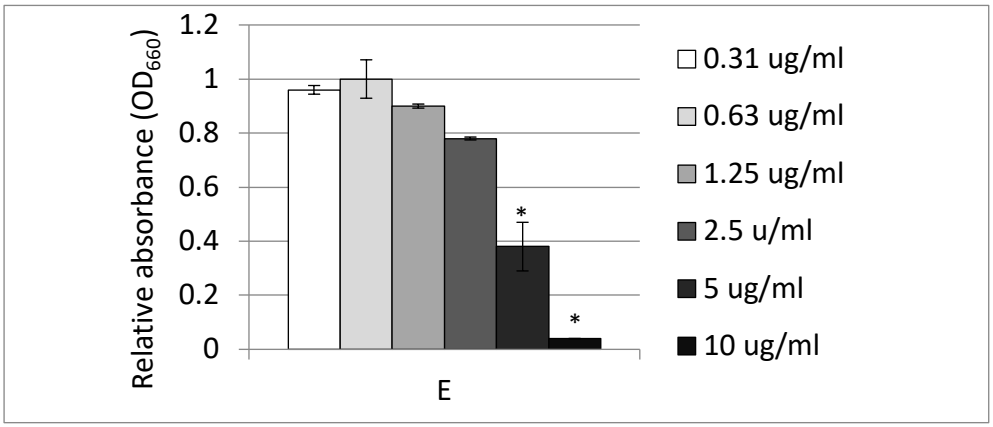

Fig. 2-10. Dose dependent of activity of ralhibitin E against *X. campestris* pv. *campestris.* The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at **P*<0.05 were considered significant.

11. Dose dependent activity of ralhibitin E against *C. michiganensis* subsp. *michiganensis*

Tomato is a key crop in global food security with estimated 169 million tons produced annually accounting for about 16% of vegetables produced (FAO, 2016). Amongst the diseases affecting tomato include bacterial wilt and canker whose causal agent is *C. michiganensis* subsp. *michiganensis* (*Cmm*) and has led to great epidemics in different growing areas globally (Blank et al., 2015; Kleitman et al., 2008; Smith, 1910; Volcani, 1985). Control has been done through the use of copper-based compounds and antibiotics in some countries (Martins et al., 2018).

The challenge facing the use of these compounds has been low efficacy especially during high disease pressure, narrow activity spectrum and development of resistance. This necessitates the development of new and more effective control compounds. Investigation into the effects of ralhibitin A-F on different bacteria species indicated that ralhibitin E could completely inhibit the growth of *Cmm* at 10 µg/ml final concentration. To further determine the MIC, a dose dependent assay was conducted. A series of ralhibitin E concentrations (10, 5, 2.5, 1.25, 0.625 and 0.31 µg/ml) were used against *Cmm* to identify the MIC of ralhibitin E. Results revealed that the MIC of ralhibitin E against *Cmm* was 10 µg/ml (Fig. 2-11). The results show that ralhibitin E can inhibit the growth of *Cmm* at a lower concentration making it a potential bactericide against the pathogen.

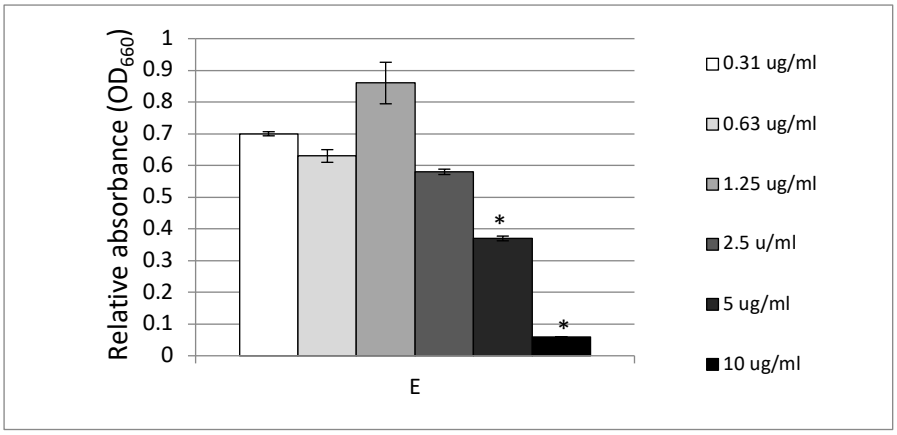

Fig. 2-11. The minimum inhibitory concentration of ralhibitin E against *Clavibacter michiganensis* subsp. *michiganensis.* The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at **P*<0.05 were considered significant.

IV. DISCUSSION

1. Identification of *R. solanacearum* growth inhibition compounds

Plant pathogenic bacterial diseases are still difficult to control due to a lack of effective bactericides. Therefore, the growth inhibitors of *R. solanacearum* one of the most important and destructive plant bacterial pathogen were investigated in this study. *R. solanacearum* is a soil borne pathogen and soil borne pathogen are difficult to control by chemicals due to the complex nature of the soil environment (Ishikawa et al., 2005). Previous chemical control method included fumigation by methyl bromide, which had shown broad activity against many pathogens in the soil. This however changed after the compound was determined to affect the

ozone layer and classified as a hazardous compound considered as class 1 of the ozone depleting substances (Ibekwe et al., 2010). This meant that this compound had to be slowly phased out up to 2005 in developed nations and 2015 in developing countries (Montreal protocol; Ajwa et al., 2003). This therefore implies that the compound with inhibitory effects against *R. solanacearum* have run out of the use period hence the need for the development of new effective compounds. This calls for development of newer and effective alternatives to control *R. solanacearum*.

Towards this aim, a screening of a chemical pilot library against *R. solanacearum* was done in this study using PDD approach. The objective was to identify compounds that could inhibit the growth of *R. solanacearum* through alteration of its phenotype. The presence of another functional group at the para position such as methyl or hydroxyl abolished their activity against *R. solanacearum*. The importance of para position of these compounds is also demonstrated by loss of activity of the chlorine (chloro) substituent in the ortho position in my study. The effective compounds were renamed as ralhibitin A (compound 1), ralhibitin B (compound 2) and ralhibitin C (compound 7). Using information from the structure activity relationship study, 3 other compounds were synthesized containing other halogens at the para position but lacking a methoxy group namely; ralhibitin D (chlorine), ralhibitin E (iodine) and ralhibitin F (fluorine). The 3 synthesized compounds were tested against *R. solanacearum* and was determined that compound D and E could inhibit the growth of *R. solanacearum* whereas compound F had poor activity. The compounds A-F are collectively referred to as ralhibitins A-F. From this study, it's clear that the position of halogens is important in ensuring activity of the ralhibitins. This is in agreement with a study that showed increased activity of 2,3-diphenyl acrylonitrile derivatives with halogens at the para position against cancerous cell lines (Li et al., 2018). Similarly, a study using cinnamylideneacetophenones confirmed that the presence of methyl or methoxy at the para position enhanced activity against *Mycobacterium tuberculosis* whereas presence of the same groups at the meta position diminished activity (Polaquini et al., 2017).

Another study has demonstrated the importance of position substituent on activity of some aldehydes (Chotsaeng et al., 2018). The compounds having halogens substituent were found to lack herbicidal activity. However, the presence of a methoxy group at a meta-position had greater efficacy when compared to the same substituent at the para position. However replacement with hydroxyl substituents determined that there was greater herbicidal efficacy when the substituent were at the para position as compared to the ortho position (Chotsaeng et al., 2018). In contrast to my study, there has also been a report of better activity of meta

substituted chlorine in nitrilise as compared to para-substituted chlorine (Jiang et al., 2016). The difference in this activity has been attributed to change in the halogen bond distribution affecting mode of substrate binding hence enhancing selectivity of the substrate (Rago et al., 2015). The presence of an halogen group in cinnamylideneacetophenones was determined to abolish activity against *Streptococcus mutans, S. sanguinis* and *Staphylococcus aureas* (Polaquini et al., 2017). However, the presence of fluorine at the para position of this compounds enhanced activity against *M. tuberculosis.*

It is also reported that isomers of meta-, para- or ortho-compounds have distinct pharmaceutical potency (Jiang et al., 2016). For example, clopidogrel and diclofenac are compounds from ortho-chlorophenylacetic and are used as anti-platelet aggregation and anti-inflammatory compounds, respectively (Bandarage et al., 2000; Bousquet et al., 2003). On the other hand, para-chlorophenylacetic acid forms baclofen and indoxacarb, which are used as a muscle relaxant and an insecticide, respectively (Li et al., 2009; Duan et al., 2014). There has also been a report of greater activity of compounds against *Fusarium graminearum* and *Valsa mali* due to the presence of electron withdrawing groups on the benzene ring (Wu et al., 2015). The presence of a bromine atom at the meta position had more potency against *F. graminearum* followed by the presence of chlorine atom at the same meta position. This indicates that against *F. graminearum*, the presence of an halogen at the meta position was important for activity (Wu et al., 2015). However, against *V. mali*, the presence of the chlorine atom at the para position had more biological potency in contrast to activity against *F. graminearum*. Contrasting results have however been reported when bromine or iodine replaced a hydrogen atom at the C8 position of flavonoids (Bahrin et al., 2014). In this study, there was decreased activity of flavonoids against *S. aureus* and *E. coli* after the introduction of a halogen atom with this phenomenon being attributed to the flavonoids size increase subsequently affecting movement across the cell membrane.

In this study, the presence of a hydroxyl group abolished the activity of compounds against *R. solanacearum*. This is in contrast to activity of cinnamylideneacetophenones where the presence of an hydroxyl group enhanced their activity against Gram-positive bacteria (Polaquini et al., 2017). However, the compounds with the hydroxyl group decreased potency against *Mycobacterium tuberculosis*. Cytotoxicity of compounds with different substitutions at position 3 of phenyl ring showed that the presence of a bulky group decreased the activity (Naito et al., 2002). Their potency was increased through the introduction of an halogen group at the phenyl ring as compared to hydroxyl group (Naito et al., 2002). Similar studies have also

been done to clarify the role of hydroxyls on the activity of phenolic compounds (Sánchez-Maldonado et al., 2011). The MIC of various phenolic compounds such as hydroxylbenzoic acid, benzoic acid, cinnamic acid were determined against *E. coli, Lactobacillus hammesii* and *L. plantarum*. The increase in the number of hydroxyl groups enhanced the activity of hydroxybenzoic acid but decreased the activity of hydroxycinnamic acids (Sánchez-Maldonado et al., 2011). Substitution of hydroxyl with a methoxy functional group was reported to increase the potency of hydroxylbenzoic acid.

My study revealed that the presence of a methoxy group was found to be not necessary in the activity of tested compounds against *R. solanacearum*. Similarly, methoxylation of cinnamylideneacetophenones was found to decrease activity against bacteria when compared to hydroxylated derivatives (Polaquini et al., 2017). Other studies have also shown the decrease in the activity of flavonoids against *E. coli* and *S. aureus* by replacement of an halogen group with a methoxy group (Bahrin et al., 2014). This reduction has been attributed to reduced transportation of the flavonoid through bacteria cell membrane due to the oxygen atom found in the methoxy acting as hydrogen bond acceptor (Bahrin et al., 2014). On the other hand, anticancer activity were decreased by the presence of methyl group on the benzene ring (Li et al., 2018).

The least concentration of a compound that can prevent visibility of bacterial growth is referred to as the MIC (Kirchhoff et al., 2018). This test is important in the determination of the right dosage of a compound in pathogen control. Determination of MIC by broth macro/micro dilution (BMD) methods is still considered the 'gold standard method' in susceptibility studies (Kirchhoff et al., 2018). This method involves incubation of standardized liquid medium volume together with standardized compound concentration against bacteria for a given period and the MIC read as the least concentration inhibiting visible bacterial growth. The ralhibitins identified in this study were active against *R. solanacearum* with a MIC of less than 10 µg/ml. The efficacy of the compounds at this low concentration makes them interesting molecules similar to another interesting compound ClCl-flav that had lower than 10 µg/ml activity against pathogens *Klebsiella pneumonia*, *Escherichia coli* and *Staphylococcus aureus* (Babii et al., 2018). The ralhibitins A, C and E were the most active against *R. solanacearum* with MIC of 1.25 µg/ml. Ralhibitin B had MIC of 2.5 µg/ml while ralhibitin D had MIC of 5 µg/ml. No antibiotic compounds have been utilized for the control of bacterial wilt disease, and no chemical compounds that specifically inhibit the growth of *R. solanacearum* at such low concentrations have been reported so far. The reported activity of antibiotics chloramphenicol

and streptomycin sulfate against *R. solanacearum* are 20 and 3.91 µg/ml respectively (Cai et al., 2018).

Novel triazole derivatives containing quinazoline moiety were shown to have significant antibacterial activity against pathogen *R. solanacearum*. The MIC against *R. solanacearum* was at 81.6 µg/ml (Fan et al., 2018). Some 1H-indol-2-ol derivatives have been shown to have inhibitory effects on both *R. solanacearum* (Tu et al., 2018). They showed a MIC of 10.25 µg/ml against *R. solanacearum* (Tu et al., 2018). My results also showed that ralhibitins are better than existing bismerthiazol (59.69 µg/ml) compounds used to control the pathogens (Cai et al., 2018). Other sulfone derivatives containing 1, 3, 4-oxadiazole moiety have also been reported to have inhibitory effect against *R. solanacearum* with MIC of 32.1 µg/ml. The effect was much greater than a commercially marketed bactericide saisentong (Copper formulation) (Xu et al., 2012). Some plant derived compounds such as coumarins have also been reported to inhibit *R. solanacearum* (Yang et al., 2016). Other natural compounds with inhibitory activity against *R. solanacearum* include resveratrol and protocatechuic acid. Amongst coumarins with activity against *R. solanacearum*, daphnetin had the greatest activity with MIC of 64 µg/ml (Yang et al., 2016). Similarly, much higher MIC have been reported for copper hydroxide, thiadizole copper, with MICs of 200 µg/ml respectively (Cai et al., 2018). Even higher concentrations at 250 µg/ml have also been reported for magnesium nanoparticles (Cai et al., 2018).

2. Species specific activity

Crop production is greatly limited by plant phytopathogenic bacteria leading to huge losses (Puigvert et al., 2018). The use of antibiotics and copper-based compounds has been an effective control strategy against bacteria from the 1900s (Elguindi, 2011; Zaumeyer, 1958). However, the emergence of resistance against agricultural bactericides is a cause for concern (Sundin et al., 2016). Previously, I identified compounds named ralhibitins A-E that could inhibit the growth of *R. solanacearum*, a Gram-negative bacterium. Further investigations showed that ralhibitins A-E could inhibit the growth of Gram-negative rice pathogen *X. oryzae* pv. *oryzae*, *Xoo*, which causes bacterial blight of rice. However, my results showed that ralhibitin E containing iodine could inhibit the growth of 2 other phytopathogenic bacteria namely; Gram-negative bacterium *X. campestris* pv. *campestris* which is responsible for black rot of diseases occurring in many cruciferous plants (Nino-Liu et al., 2006; Vicente and Holub,

2013) and Gram-positive bacterium *C. michiganensis* subsp. *michiganensis* (*Cmm*), which is a causal agent of bacterial canker of tomato (Chalupowicz et al., 2012) at 10 µg/ml.

Control strategies against *X. oryzae pv. oryzae* include the use of chemicals, bio-control and breeding for resistance (Kim et al., 2016). For many years, the compounds Bismethiazol and Zinc thiazole have been used to control bacterial blight in rice (Liang et al., 2015). The thiadizole compounds control bacterial blight through inhibition of growth. Examples of biological control include the utilization of rhizobacteria formulations, the use of *Pseudomonas* and *Bacillus Species* (Chithrashree et al., 2011; Gnanamanickam, 2002). Currently, there are over 30 compounds that have been reported to have inhibitory activity against bacterial blight such as antibiotic streptomycin (Sahu et al., 2018). However, the compounds have been shown to be expensive and with residual effects on plants and this calls for identification of other better alternatives (Islam et al., 2016). Results from my study indicate that ralhibitins (A-E) had inhibitory effect on *X. oryzae* pv. *oryzae*. The most active compound, ralhibitin D had a MIC of 5 µg/ml. Recently, 2-mercapto-5-substituted-1,3,4-oxadiazole/thiadiazole derivatives were reported to have antibacterial activity against *Xoo*, and the half-maximal effective concentration value was 14.69 µg/ml against *Xoo* (Li et al., 2015).

Screening of human drugs as antibacterial agents against bacterial blight identified a compound, niclosamide to be effective against the pathogen (Kim et al., 2016). The compound was able to inhibit bacterial growth *in vitro* and also blocked the proliferation of the disease in infected rice plants (Kim et al., 2016; Sahu et al., 2018). The MIC of niclosamide against bacterial blight strains (10208, K3a, PXO99) was 5 µg/ml. However, the compounds could not inhibit the growth of other tested bacteria such as *E. coli* strains (DH10b, Top 10 and Rosseta2) (Kim et al., 2016). Therefore, ralhibitin D can be considered as potential bactericide together with niclosamide as they have similar activity. Some plant based extracts such as *Terminalia chebula, Anethum graveolens* and *Ferula assa-foetida* have also shown potency against bacterial blight in rice (Jabeen, 2011). Similarly, a report has shown the activity of sulfone derivatives against *X. oryzae* pv. *oryzae* (Shi et al., 2015). The best sulfone compound 2-(methylsulfonyl)-5-(4-fluorobenzyl)-1,3,4-oxadiazole had a MIC of 9.89 µg/ml (Shi et al., 2015). The MIC for existing commercial compounds has been pegged at 92.61 µg/ml for bismerthiazol and 121.82 µg/ml for thiadizole copper respectively (Shi et al., 2015). Some quinazolin derivatives have also been reported to inhibit the growth of *Xoo* with the most active compound having MIC of 28.2 µg/ml (Lv et al., 2017). The MIC for ralhibitins in this study against *X. oryzae* pv. *oryzae* was 5 µg/ml which is much lower than most of the commercial

products and other reported compounds.

Investigation showed that ralhibitin E could inhibit the growth of *C. michiganensis* subsp. *michiganensis* at a final concentration of 10 µg/ml. Further, dose dependent studies were done and determined that the MIC of ralhibitin E against *C. michiganensis* subsp. *michiganensis was* 10 µg/ml. Some bactericides such as bronopol, oxolinic acid, oxytetracycline, streptomycin, and 8-hydroxyquinoline were investigated against *Cmm*, and their MIC were determined to be between 4-8 µg /ml (de León et al, 2008). Despite this progress, there are currently no chemicals to comprehensively control the disease (de Leôn et al., 2008). Therefore, ralhibitin E may be another candidate for this bactericide. Some essential oils namely; rosemary, dictamnus, lavender, thyme, marjoram and sage have been reported to inhibit the growth of *C. michiganensis* subsp. *michiganensis* at a MIC of 85-300 µg/ml (Daferera et al., 2003). Other compounds such as drimanic compounds namely; drimenol, polygondial, nordrimenone and isonordrimenone have also been reported to have activity to inhibit the growth of *Clavibacter michiganensis* subsp. *michiganensis* (Montenegro et al., 2018). Against *C. michiganensis* subsp. *michiganensis,* the most active compound Polygodial had a MIC of 16 µg/ml.

There is no effective method to control *Xanthomonas campestris* pv. *campestris* (*Xcca*) however, control strategies like the use of zinc sulphate, sodium hypochlorite, antibiotics, and seed treatments have been used (Vicente and Holub, 2013). Control of *X. campestris* pv. *campestris* of cabbage by hot air has been reported in Japan (Shiomi, 1992). Treatment with 75°C eliminated the pathogen after 7 days in seeds artificially infected with the pathogen whereas the disease was eliminated at 75°C for two days under natural disease infection (Shiomi, 1992). Breeding for resistance against black rot has been attempted as it is environmentally safe and cheaper as compared to the use of chemicals (Taylor et al., 2002). In this study we identified ralhibitin E with inhibitory effect against *X. campestris* pv. *campestris*. The compound had a MIC of 10 µg/ml. Some soil microorganisms have been shown to control the growth of *Xcc* (Rafael et al., 2018). Filtrates from *Paenibacillus* (TCDT-08) showed inhibitory activities against *Xcc* with MIC of 128 µg/ml (Rafael et al., 2018). Recently there has been a report showing the control of black rot using bio-control (Nuñez et al., 2018). The use of bio fertilizer and milk-based components significantly reduced the severity of the disease. The MIC of some medicinal plants against black rot was reported to be 32 µg/ml (de Britto et al., 2011). Screening of some peptides from *Pseudonocardia endophytica* VUK-10 identified one compound that could inhibit the growth of *Xcc* at MIC of 8 µg/ml (Mangamuri et al., 2016). Some antibiotics have also been screened against *Xcc* and determined that the most active was

cholertetracycline at 200 µg/ml (Bhat and Masoodi, 2000). However, others like carbendazim (1000 µg/ml), ampicillin (200 ug/ml), mancozeb (1500 µg/ml), penicillin (200 µg/ml), zineb (1500 µg/ml) were not active against the pathogen (Bhat and Masoodi, 2000). Therefore, ralhibitin E is a potential candidate bactericide against *X. campestris* pv. *campestris* as its active at a very low concentration compared to reported bactericides.

In this study, ralhibitin E has been determined to control both Gram-negative (*Rs, Xoo, Xcc*) and Gram-positive bacterium (*Cmm*). Ralhibitin E can be used in the production of a broad-spectrum bactericidal candidate due to its wide spectrum of activity. The advantage of broad spectrum compounds is that they can provide intermediate control to disease epidemics that have not been characterized (Liebens et al., 2014). Ralhibitin E is an analogue of ralhibitin A obtained by replacement of bromine with iodine. The halogen bond (X-bond) strength has been found to be influenced by the σ-hole properties (Fanfrlík et al., 2015). Increase in atomic size of halogens directly results to increase in σ-hole being greatest in iodine. Therefore, a replacement of chlorine by iodine has been reported to enhance the activity in capthesin-inhibitor complex (hardegger et al., 2010). The broad activity of the iodine substituent in ralhibitin E is likely therefore to be because of the effect of the σ-hole. Iodine was reported to have desirable biocidal properties manifesting as rapid virucidal, fungicidal, sporicidal, and bactericidal effects (Zubko and Zubko, 2013). This may explain the broad activity range of ralhibitin E against both Gram-negative and Gram-positive bacteria in this study. It is also reported that iodine had a higher activity against *S. aureas* and *Pseudomonas aeruginosa*, and that the bacterial biofilm was completely eliminated within 24 h after treatment (Thorn et al., 2009). A similar study on the effect of halogen substituents on activity of flavonoids against *Staphylococcus aureus* revealed similar results (Bahrin et al., 2016). In this study flavonoids containing fluorine, chlorine, bromine and iodine had a MIC of 0.97-7.81 µg/ml whereas flavonoids with a hydrogen substituent recorded a MIC of 62.5 µg/ml. Amongst the halogen replacements, the greatest activity was recorded for iodine at MIC of 0.97 µg/ml against *S. aureus* and 31.17 µg/ml against *Escherichia coli* respectively (Bahrin et al., 2016).

The pathogens *Rs, Xcc, Xoo* and *Cmm* are collectively referred to as vascular bacteria (Bae et al., 2015). This is because despite their different infection roots (*Rs*), hydathodes (*Xoo, Xcc*) or stem (*Cmm*), their destination has been confirmed to be the xylem vessels (Mansfield et al., 2012; Bae et al., 2015). Xylem vessels functions are disrupted by the action of these bacteria to release large quantities of exopolysaccharides (EPS) and formation of biofilms on the xylem

vessels walls (Bae et al., 2015). The net effect of this EPS and biofilm deposition is the blockage of movement of water through the xylem vessels leading to wilting of plants. Due to the fact that all the pathogens inhibited by ralhibitins colonize the xylem tissues, then its highly possible that the target sites could be the substances in bacteria that target the xylem tissues such as the biofilms and EPS.

Ralhibitins in my study were more active against Gram-negative bacteria as compared to Gram-positive bacteria. This is in contract to a flavonoid compound ClCl-flav that was more active against Gram-positive bacteria than the Gram-negative bacteria with the reason for this behavior linked to the presence of high amounts of peptidoglycan in Gram-negative bacteria than Gram-positive bacteria (Babii et al., 2018). Our results therefore suggest that our compounds were able to overcome the peptidoglycan barrier in the cell membrane of Gram-positive bacteria hence greater inhibitory activity.

3. Stability of ralhibitins

The factors that affect activity of compound include light, temperature, pH and test medium composition (Paesen et al., 1994). For example, a study has shown that medium has an influence on the stability of compounds and the reason for this changes have been attributed to the nature of medium being either lipophilic or aqueous (Cielecka-Piontek et al., 2015). Some antibiotics like amoxicillin are affected by temperature and pH. The amoxicillin storage temperature range is (2–8 °C) and inappropriate condition renders them ineffective due to degradation (Naidoo et al., 2006). It has been shown that antibiotics clarithromycin and amoxilicillin stability occurs at neutral pH 7. Other reports indicate that it's difficult for extrapolation of compound stability in one condition with another (Erah et al., 1997). In our study, ralhibitins were found to be stable over a wide range of temperature and pH. These properties are important in the storage and distribution of ralhibitins especially in the developing countries with a hot climate and lacking refrigeration facilities. Because ralhibitins are highly stable, they have much advantage for application.

There is a need to determine the bactericidal effects of compound at different pH ranges (Wiegand et al., 2015). The active negative logarithm of hydrogen ions when present in aqueous solutions in expression of alkalinity or acidity is referred to as pH and has a scale of (0-14) (Wiegand et al., 2015). In my study, ralhibitins (A-E) were determined to be stable at pH 7 and 9. However, the activity for ralhibitins A-D was slightly decreased at pH 6 while the

activity of ralhibitin E was not affected at this pH. In agreement with my results, a report has shown that the activity of antibiotics is pH dependent (Wiegand et al., 2015). This report showed greatest influence of pH on gentamycin and polihexanide. Against *S. aureus*, there was an increase in activity of polihexanide at pH ranges 5.0-9.0. Similar result showing an increase in efficacy for polihexanide was seen against *P. aeruginosa* at pH 7.0-9.0. The reason for the increase in potency at high pH has been attributed to polycationic state of polyhexanide (Wiegand et al., 2015). At pH 7, there is binding between the negative charge of bacteria and polyhexanide positive charge resulting to bacterial membrane destruction and eventual bacterial death (Ikeda et al., 1983).The effect of gentamycin on bacteria at low pH is due to the degradation of the antibiotic due to the damage caused to its trisaccharide structure by acid (Wiegand et al., 2015). The pH of test medium has been found to affect the MIC of fluconazole against *Candida* species (Danby et al., 2012). When the test medium became acidic (low pH), there was an increase in MIC of fluconazole. An anti-dermatophyte compound ciclopirox olamine has been reported to also increase the MIC by four folds after a decrease in test medium pH (Danby et al., 2012). An assay to determine the pH effect on antibacterial activity of some antiseptics revealed that the activity of octenidine and chlorhexidine against bacteria was independent of pH (Wiegand et al., 2015). Their effect of pH on antiseptic activity was also determined to be bacteria specific. For example, activity of silver nitrate against *P. aeruginosa* was determined to decrease with increasing pH while that against *S. aureus* increased (Wiegand et al., 2015). Similarly, increasing pH decreased the antibacterial activity of PVP-iodine. Reports have shown that PVP-iodine a compound with broad activity spectrum is active only up to pH 7 (Ratiopharm, 2008).

My study revealed that ralhibitins were stable at both low and very high temperatures. They were able to retain their activity after exposure to changing temperature regimes. However, contrasting results have been reported for some antibiotics namely; piperacillin, ampicillin, azlocillin, cefotaxime, moxalactam, cefazolin, imipenem and ticarcillin have been stored at different temperatures 4°C, -10°C, -25°C, and -70°C and then were used for susceptibility studies against *Eschelichia coli* and *Staphyloccus aureus* (Nickolai et al., 1985). Interesting results were obtained whereby the antibiotics were more stable at -70°C > -25°C >4°C >-10°C respectively. The decrease in stability of the compounds at -10°C was attributed to fluctuation in potency during the freezing and thawing of the compounds. Antibiotics cefoperazone and cefazolin were determined to be highly stable at all the temperatures (Nickolai et al., 1985). However, the remainder of the antibiotics had different levels of degradation at all temperatures except at -70°C which was subsequently recommended to be used for storage of the compounds.

Other studies have also shown degradation of antimicrobial compounds at various storage temperatures with lower temperatures of -70° C considered the best for compound storage (Lallemand et al., 2016). There have been differences in storage stability in compounds of the same class such as meropenem and imipenem (Nickolai et al., 1985;Dowzicky et al., 1994)These studies determined that meropenem was highly stable in similar conditions as compared to imenepem. This implies that each compound should always be subjected to stability tests before deciding their storage conditions. Degradation of compounds brought about by storage conditions has been reported to have an effect on the MIC. The storage duration and degradation increased the MIC values (White et al., 1991). Another similar study also confirmed the increase in MIC values as influenced by degradation of compounds (Lallemand et al., 2016).

On the other hand, fungizone was reported to have the least shelf life in activity when exposed to freezing conditions. This results therefore helped peg the shelf life of the antibiotic mixture at 1 week at -30 °C or -80 °C. Bacterial biofilms have been significantly reduced by antibiotics incubated at different temperatures (35 °C, 40 °C and 45 °C respectively (Hajdu et al., 2010). Higher temperature incubation of antibiotics of 40 °C or 45 °C had a significantly greater effect of biofilms reduction as compared to lower temperature of 35 °C. Therefore, slight increase in the temperature enhanced the activities of antibiotics against biofilms but complete eradication of the pathogen was not possible. A study on the antibacterial activity of silver molecules revealed that stability was important for their activity (Aldabaldetrecu et al., 2018).

Another study investigating the role of freezing and storage temperatures of antibiotics (tobramycin and fungizone) on the antibacterial activity reported that their stability was affected by low temperatures (Mirabet et al., 2018). A previous examination of antibiotic mixture containing tobramycin, vancomycin, fungizone and co-trimoxazole at 4 °C reported that antimicrobial activity lasted for 6 weeks (Holder and Robb, 1998;1999). Allicin compounds obtained from garlic have been reported to have differing antibacterial activities after temperature treatment (Fujisawa et al., 2008). For instance, at 4 °C, the antibacterial activity half-life against *Escherichia coli* was 26 days while for *Staphylococcus aureus* was 14 days. On the other hand, at 37 °C, the antibacterial activity half-life was 1.9 and 1.2 days for *E. coli* and *S. aureus*, respectively. This showed a decrease in potency of allicin on exposure to higher temperature ranges. The efficacy of some disinfectants (chlorhexidine, acetate, sodium hypochlorite and glutaraldehyde) has also been shown to increase after exposure to varying temperatures (4 °C, 20 °C, 37 °C, 50 °C) (Gelinas et al., 1984). Comparatively, sodium

hypochlorite and iodophor were less affected by temperature when compared to glutaraldehyde. Majority of the compounds had greatest activity at 37 °C. However, the bactericidal effect of compounds at temperatures below the room temperature was drastically reduced with chlorhexidine being greatly affected (Gelinas et al., 1984).

V. MATERIALS AND METHODS

1. Identification of *R. solanacearum* inhibiting compounds

R. solanacearum in glycerol stock and stored at -80 C was streaked on BG (Bacto peptone 10 g, yeast extract 1 g, casamino acid 1 g/L water, pH 7) agar plates and incubated at 27° C overnight. Then a colony was dropped in 3 ml BG liquid medium and incubated overnight. Overnight cultured *R. solanacearum* (*Rs*1002) (Mukaihara *et al.* 2004) in BG medium was harvested by centrifugation and suspended in fresh BG medium to adjust OD_{600} = 0.1. One hundred fifty µl of bacterial suspension was aliquot into each well of 96 microtiter plate. Into each well, 0.5 µl of each chemical (3 mg/ml in DMSO) was added to examine the effect of bacterial growth. As a control the same amount of DMSO was also added. Microtiter plate was incubated for 24 hr at 27 °C in DeepWell Maximizer (TAITEC, Koshigaya, Japan), then absorbance at OD_{600} was measured by iMARK Microplate Reader (BIO-RAD, Tokyo, Japan). To confirm the reliability of this assay system, we checked the growth inhibitory effect of the compounds in the authentic library (80 compounds) provided by the antibiotics laboratory RIKEN Japan. Among authentic library, there are antibiotics such as novobiocin, rifampicin, spiramycin I, neomycin B, streptomycin, tetracycline hydrochloride and bicyclomycin.

2. Determination of active functional group in compound 1

To determine the active structure in the pharmacophore of active compound, different chemicals were purchased. Compound one had some substituents and functional groups such as a halogen group (bromine), presence of a methoxy group which could be modified in order to find out the active pharmacophore. Eight compounds plus the active compound were purchased with some modifications to compound 1. The modifications included, introduction of other functional groups such as hydroxyl (OH), methyl (CH_3), other halogens (Cl, F) in place of bromine, removal of the methoxy group, change of location of halogen group (from para to ortho), introduction of an extra benzene. The compounds were used to determine their antimicrobial activity against *R. solanacearum* at a final concentration of 10 µg/ml.

3. Synthesis of compound 1 analogues

3.1 Synthesis of Fluorine derivative (ralhibitin F)

To synthesize ralhibitin F, a mixture of tryptamine (1.8 g, 13 mmol) and 4-fluorobenzaldehyde (1.9 g, 10 mmol) in acetic acid (50 ml) was reacted at 100 °C for 20 h. The reaction mixture was added to H_2O (100 ml), and the suspension was extracted with ethyl acetate (AcOEt), washed with brine, and recrystallized. The precipitate was filtered, washed with AcOEt and hexane, and dried under vacuum to obtain a pale yellow powder (1.6 g).

3.2 Synthesis of bromine derivative (ralhibitin C)

To synthesize ralhibitin C, a mixture of tryptamine (1.8 g, 13 mmol) and 4-bromobenzaldehyde (1.9 g, 10 mmol) in acetic acid (50 ml) was reacted at 100 °C for 20 h. The reaction mixture was added to H2O (100 ml), and the suspension was extracted with ethyl acetate (AcOEt), washed with brine, and recrystallized. The precipitate was filtered, washed with AcOEt and hexane, and dried under vacuum to obtain a pale yellow powder (1.6 g).

3.3 Synthesis of Iodine derivative (ralhibitin E)

To synthesize ralhibitin E, a mixture of tryptamine (1.8 g, 13 mmol) and 4-iodobenzaldehyde (1.9 g, 10 mmol) in acetic acid (50 ml) was reacted at 100 °C for 20 h. The reaction mixture was added to H_2O (100 ml), and the suspension was extracted with ethyl acetate (AcOEt), washed with brine, and recrystallized. The precipitate was filtered, washed with AcOEt and hexane, and dried under vacuum to obtain a pale-yellow powder (1.6 g).

4. Effect of synthesized analogues of compound 1 on growth of *R. solanacearum*

R. solanacearum was cultured from a glycerol stock stored in a freezer at -80 C overnight at 27° C. Further, the bacteria were grown overnight in 3 ml BG medium at 27° C, 200 rpm shaking for 24 hours. The 10 µl of bacterial suspension was diluted into fresh 3 ml medium and shaking briefly. Then the ralhibitin C, ralhibitin E and ralhibitin F were added to 3 ml of medium at a final concentration of 10 µg/ml and incubation was done for overnight. DMSO was used as a control. The bacterial absorbance at OD_{660} was measured.

5. Dose dependent activity of compound 1 and its analogues against *R. solanacearum*

Compound 1 and it's analogues (here in referred to as ralhibitins A-F) were determined to completely inhibit the growth of *R. solanacearum* at a final concentration of 10 µg/ml. The ralhibitins A, B, C, D, E and F were evaluated for antibacterial activity using broth macro dilution method in order to determine their MIC. *R. solanacearum* was cultured from a glycerol stock stored in a freezer at -80 C overnight at 27° C. Further, the bacteria were grown overnight in 3 ml BG medium at 27° C, 200 rpm shaking for 24 hours. Then two-fold serial dilution in 3 ml BG medium was done by first pipetting 10 µl of bacterial suspension to fresh 3 ml medium and shaking briefly. Then 3 µl of the diluted bacterial suspension was pippeted to fresh 3 ml medium to which ralhibitin A, B, C, D, E and F was added (10mg/ml). Different final concentrations of 0.31, 0.63, 1.25, 2.5, 5 and 10 µg/ml were then added to the bacterial suspension and incubation was done overnight for 24 hours. DMSO was used as a negative control. The bacterial absorbance at OD_{660} was measured. The MIC was the least concentration of the compound that inhibited the growth of bacteria after 24 hours incubation at 27°C rotary shaker.

6. Thermostability

R. solanacearum strain *Rs*1002 stored at -80 °C was streaked on BG (Bacto peptone 10g, Yeast extract 1 g, casamino acid 1 g) plates and incubated at 27 °C overnight. Then a colony was obtained from the overnight culture and grown in 3 ml BG tubes containing liquid medium overnight shaking at 27 °C to mid-log phase. Ralhibitins stock solutions were prepared by dissolving 10 mg of powder in 1 ml of DMSO forming the working stock solution. An aliquot of 50 µl of each ralhibitin was then kept at 4°C, 37 °C, 50°C for 24 hours. Another aliquot was subjected to autoclaving at 121°C. To determine the effect of temperature on activity of ralhibitins, 10 µl of *Rs*1002 from liquid culture obtained from overnight liquid culture was suspended in fresh 3 ml BG liquid medium and mixed briefly. Then 3 µl of this bacterial suspension was obtained and mixed with fresh 3 ml BG liquid medium in test tube as further dilution. Ralhibitins treated at different temperatures were then added to make 10 µg /ml final concentration. Control samples were treated with ralhibitins in DMSO. After 24 hours, bacteria absorbance at OD_{660} was measured. The experiment was conducted in 3 replicates and repeated three times.

7. pH Assay

The growth inhibition by ralhibitins at different pHs in BG medium was investigated. *R. solanacearum* strain *Rs*1002 stored at -80 °C was streaked on BG plates and incubated at 27 °C overnight. Then, the pH of the BG medium was adjusted to 6.0,7.0, or 9.0 by the addition of HCl or KOH. Then a colony was obtained from the overnight culture and grown in 3 ml BG liquid medium of different pH values (pH 6, pH 7 and pH 9) in test tubes overnight shaking at 27 °C to mid-log phase. To determine the effect of pH on activity of ralhibitins, 10 µl of *Rs*1002 from BG liquid culture at different pH values (6, 7 and 9) obtained from overnight liquid culture was suspended in fresh 3 ml BG liquid medium of the respective pH values and mixed briefly. Then 3 µl of this bacterial suspension was obtained and mixed with fresh 3 ml BG liquid medium of different pH values (6, 7 and 9) in test tube as further dilution. Then ralhibitins (A-F) from 10mg/ml stock solution were added to the tubes to a final concentration of 10 µg/ml. Control samples were treated with ralhibitins in DMSO at the respective pH values of medium. After 24 h incubation at 27 °C, absorbance at OD_{660} was measured. The experiment was conducted in replicates of three and repeated three times.

8. Killing assay of ralhibitin E

R. solanacearum strain *Rs*1002 stored at -80 °C was streaked on BG plates and incubated at 27 °C overnight. Then a colony was obtained from the BG plate and grown in 3 ml BG liquid medium test tubes overnight shaking at 27 °C to mid-log phase. Then 10 µl of each bacteria suspension was added to 3 ml distilled water followed by addition of 3 µl of ralhibitin E making a final concentration of 10 µg/ml. Control tubes had 10 µl of *R. solanacearum* without addition of ralhibitin E. Then aliquots of the bacterial suspension were picked at intervals of 0, 6, 12, 24 and 48 hours in order to determine whether the compounds inhibit or kill the bacteria. Serial dilutions of the bacterial aliquots were done. The aliquots were spread on BG nalixidic acid (50 µl/ml) and incubated at 27° C. Bacterial numbers were quantified by using CFU/ml.

9. Species specific activity of ralhibitins against phytopathogenic bacteria

9.1 Bacterial strains used

Table 9.1. Bacterial strain and their growth medium used in this study

Bacterial name	(For *R. solanacearum*)			Abbreviation	Medium	Source
	race	biovar	phylotype			
*Ralstonia solanacearum Rs*10002	1	4	I	Rs1002	BG	Mukaihara et al. 2004
R. solanacearum OE1-1	1	4	I	RsOE-1	BG	Mori et al. 2016
R. solanacearum 6601	1	3	I	301070	BG	MAFF301070
R. solanacearum 970825-11	4	4	I	211479	BG	MAFF211479
R. solanacearum POPS 8409	3	N2	IV	211271	BG	NAFF211271
Acidovorax avenae NARCB200109, T13052				*Aa*	NA	MAFF106618
Burkholderia glumae Pg-10				*Bg*	YP	Maeda et al. 2007
Chromobacterium violaceum Cv026				*Cv*	LB	McClean et al. 1997
Clavibacter michiganensis subsp. *michiganensis* 05M1-2				*Cmm*	LB	Okayama Pref.
Dickeya dadantii 92-31				*Dd*	KB	MAFF311041
Escherichia coli DH5				*Ec*	LB	Nippongene
Pantoea ananatis NARCB200120 AZ200124				*Pa*	LB	MAFF106629
Pectobacterium carotovorum subsp. *carotovorum* EC1				*Pcc*	YP/KB	Hossain & Tsuyumu 2006
Pseudomonas protegens Cab57				*Pp*	KB	MAFF212077
P. syringae pv. *tomato* DC3000				*Pto*	KB	Higashi et al. 2008
Rhizobium radiobacter (Ti) GV3101 (pMP90)				*Rr*	LB	Clough and Bent 1998
Xanthomonas campestris pv. *campestris* XcA				*Xcca*	Moka Rm	MAFF301151
X. campestris pv. *citri* NS387				*Xcci*	NA	MAFF311001
X. oryzae pv. *oryzae* T7174				*Xoo*T	KB	MAFF311018
X. oryzae pv. *oryzae* H-9101				*Xoo*H	KB	MAFF210548
X. oryzae pv. *oryzae* KXO 93-1				*Xoo*K	KB	MAFF210893
X. oryzae pv. *oryzae* T7147				*Xoo*	KB	MAFF301226

The composition of bacterial media was BG medium (Bacto peptone 10 g, yeast extract 1 g, casamino acid 1 g/L water, pH 7.0), LB medium (Bacto tryptone 10 g, yeast extract 5 g, NaCl 7 g/L water), YP medium (Bacto peptone 20 g, yeast extract 10 g/L water), KB medium (King et al. 1954) and Moka Rm medium (yeast extract 4 g, casamino acid 8 g, K_2HPO_4 2 g, $MgSO_4$ 0.3 g/L water). NA (Bacto peptone 5g, Yeast extract 3g, NaCl 5g/L water)

9.2 Species specific activity of ralhibitins against phytopathogenic bacteria assay

Bacteria used in this study were cultured on their respective growth medium (Table 9.1) from a glycerol stock stored in a freezer at -80 C overnight at 27° C. Further, the bacteria species was grown overnight in 3 ml of respective medium at 27° C, 200 rpm shaking for 24 hours. Then two-fold serial dilution in 3 ml respective medium was done by first pipetting 10 µl of bacterial suspension to fresh 3 ml medium and shaking briefly. Then 3 µl of the diluted bacterial suspension was pippeted to fresh 3 ml medium to which ralhibitin A-F were added to

a final concentration of 10 µg/ml and incubation done overnight for 24 hours. DMSO was used as a control. The bacterial absorbance at OD_{660} was measured.

9.3 Dose dependent activity against *X. oryzae* pv. *oryzae*

Compounds named as ralhibitins were determined to completely inhibit the growth of *X. oryzae* pv. *oryzae* at a final concentration of 10 µg/ml. The ralhibitins A-F were evaluated for antibacterial activity using broth macro dilution method in order to determine their MIC. *X. oryzae* pv. *oryzae* was cultured on KB medium from a glycerol stock stored in a freezer at -80 C and incubated overnight at 27° C. Further, the bacteria were grown overnight in 3 ml KB medium at 27° C, 200 rpm shaking for 24 hours. Then two-fold serial dilution in 3 ml KB medium was done by first pipetting 10 µl of bacterial suspension to fresh 3 ml medium and shaking briefly. Then 3 µl of the diluted bacterial suspension was pippeted to fresh 3 ml medium to which ralhibitin A, B, C, D, E and F was added (10mg/ml). Different final concentrations of 0.31, 0.63, 1.25, 2.5, 5 and 10 µg/ml were then added to the bacterial suspension and incubation done overnight for 24 hours. DMSO was used as a negative control. The bacterial absorbance at OD_{660} was measured. The MIC was the least concentration of the compound that inhibited the growth of bacteria after 24 hours incubation at 27° C rotary shaker.

9.4 Dose dependent activity against *X. campestris* pv. *campestris* and *C. michiganensis* subsp. *michiganensis*

Ralhibitin E was determined to completely inhibit the growth of *X. oryzae* pv. *oryzae* at 10 µg /ml final concentration. Further, the MIC of ralhibitin E against *X. campestris* pv. *campestris* (Xcca) was investigated. *Xcca* and *Cmm* were cultured on Moka Rm and LB medium, respectively, from a glycerol stock stored in a freezer at -80 C and incubated overnight at 27° C. Further, the bacteria were grown overnight in 3 ml of each respective liquid medium at 27° C, 200 rpm shaking for 24 hours. Then two-fold serial dilution in 3 ml each medium was done by first pipetting 10 µl of bacterial suspension to fresh 3 ml medium and shaking briefly. Then 3 µl of the diluted bacterial suspension was pippeted to fresh 3 ml medium each medium to which ralhibitin E was added at different final concentrations of 0.31, 0.63, 1.25, 2.5, 5, 10 µg/ml and incubation done overnight for 24 hours. DMSO was used as a negative control. The bacterial absorbance at OD_{660} was measured. The MIC was the least concentration of the compound that inhibited the growth of bacteria after 24 hours incubation at 27° C rotary shaker.

CHAPTER III

In vivo Control of *Ralstonia solanacearum* in Tomato

I. SUMMARY

In my previous study, I identified compounds known as ralhibitins (A-E), that could control the growth of *Ralstonia solanacearum* in *in vitro* with minimum inhibitory concentrations ranging between (1.25-5 µg /ml). Therefore, I investigated *in planta* activity of ralhibitin D, which is soluble in ethanol to protect tomato seedlings against *R. solanacearum.* Disease symptoms was scored as disease indexes, and the degree of disease suppression was evaluated as control efficiency, which was obtained by the formula 100(C-T)/C, in which C and T are disease indexes of control group and ralhibitin-treated group, respectively. As a result, ralhibitin D significantly suppressed tomato bacterial wilt in a concentration dependent manner. The control efficiency was 96.3 at 9 days post inoculation (DPI) with 111 µg/ml of ralhibitin D, and 87.7 at 10 DPI with 56 µg/ml of ralhibitin D under lower bacteria concentration (OD_{660nm} = 0.06). Disease suppression was also observed at higher bacterial concentration (OD_{660nm} = 0.2). The control efficiency was 75.4 at 10 DPI with 111 µg/ml concentration of ralhibitin D, and 69.3 at 10 DPI with 56 µg/ml concentration. Based on these findings, ralhibitin D can be a potential bactericide to control bacterial wilt in tomato.

II. INTRODUCTION

For practical applicability of active compounds against plant pathogens identified in vitro, their *in planta* studies are important. Several studies have reported compounds with *in vitro* growth inhibition to *R. solanacearum*. For instance, a study has demonstrated that some hydroxycoumarins namely daphnetin, esculetin and umbelliferone have growth inhibitory effects to *R. solanacearum* (Yang et al., 2018). A further study was done to investigate the *in planta* activity of the hydroxycoumarins against *R. solanacearum* and revealed that the control efficiency at 10 days post inoculation (DPI) was 59.19 for daphnetin, making this compound a potential protective agent against the pathogen. Similarly, another study found that protocatechualdehyde had *in vitro* growth inhibition against *R. solanacearum* and further investigated it's *in planta* activity (Li et al., 2016). The compound had control efficiency of 92.01 at 9 DPI.

Different inoculation methods are used to study virulence and pathogenicity mechanisms of soil borne pathogen *R. solanacearum* such as stem inoculation, soil drenching, petiole cut and leaf clipping (Singh et al., 2018). *R. solanacearum* infects hosts through the roots hence the use of other methods of inoculation other than soil drenching has been reported to have a different pathogen behavior inside the plant (Singh et al., 2018). Also, the tomato plant has

been commonly utilized as a model host especially when the pathogen mode of inoculation is through the stem or by soil drenching. However, other model plants have been utilized in studying the virulence of *R. solanacearum* such as *Phaseolus vulgaris, Mimosa pudica* and *Arabidopsis thaliana* (Sing et al., 2018). Adult plants have been used in *R. solanacearum* assays but have been limited by huge economic investments and utilization of huge space. There have also been reports of the use of seedlings for studying *R. solanacearum* pathogenicity (Macho et al., 2010). Recently, an innovative inoculation method using tomato seedlings in microfuge tubes has been reported with symptom development occurring 48 hours post inoculation (Singh et al., 2018). In my previous study, I identified compounds named ralhibitins A-E that completely inhibited the growth of *R. solanacearum* i*n vitro*. Therefore, determining the efficiency of these compounds *in planta* is necessary for their future potential applicability in the natural conditions to control *R. solanacearum*.

III. RESULTS

1. Ralhibitin D protects tomato seedlings from *R. solanacearum* infection at lower bacteria concentration (OD_{660nm} = 0.06)

To determine whether the ralhibitins can protect infected tomato seedlings from *R. solanacearum*, I conducted an *in planta* assay. Tomato plants were used because they are considered model plants for understanding the virulence and behavior of *R. solanacearum* (Singh et al., 2018). I used tomato variety Ponderosa that is susceptible to the pathogen *R. solanacearum* pathogen (*Rs*1002) for the *in planta* experiment. *Rs*1002 which is a nalixidic acid resistant derivative of *Rs*1000 was used in this study. In my study I used the soil drenching method whereby the pathogen is inoculated through irrigation of the roots of tomato seedlings. This is because this mode of pathogen infection of plants is used by *R. solanacearum* in the natural environment as the disease is soil borne. The utilization of seedlings in my experiments was informed by other studies that have reported ease of studying pathogenicity of *R. solanacearum* in seedlings as compared to adult plants (Singh et al., 2018).

A preliminary study confirmed pathogenicity of the *R. solanacearum* in infected tomato seedlings (Data not shown). Strain *Rs*1002 at a lower bacterial population (OD_{660nm} = 0.06) and ralhibitin D at 111 µg/ml were drenched into the soil of tomato seedlings and disease severity observed by the number of wilting plants. Tomato seedlings inoculated with only *R. solanacearum* at lower bacterial population acted as control group. Notable wilting of plants in the control group was observed 6 DPI in control tomato seedlings. There were significant differences ($p<0.05$) in disease index between the control without ralhibitin D and the treatment with ralhibitin D at this concentration (Fig. 3.1a). The disease index for the control was 17.4 at

6 DPI while the treatment recorded a disease index of 0.6. At 9 DPI, the disease index of the control increased remarkably to 51.7 while that of ralhibitin D treated seedlings reached 2.7. The control efficiency at 111 µg/ml was determined to be 96.9% at 6 DPI and 96.3% at 9 DPI (Fig. 3.1b).

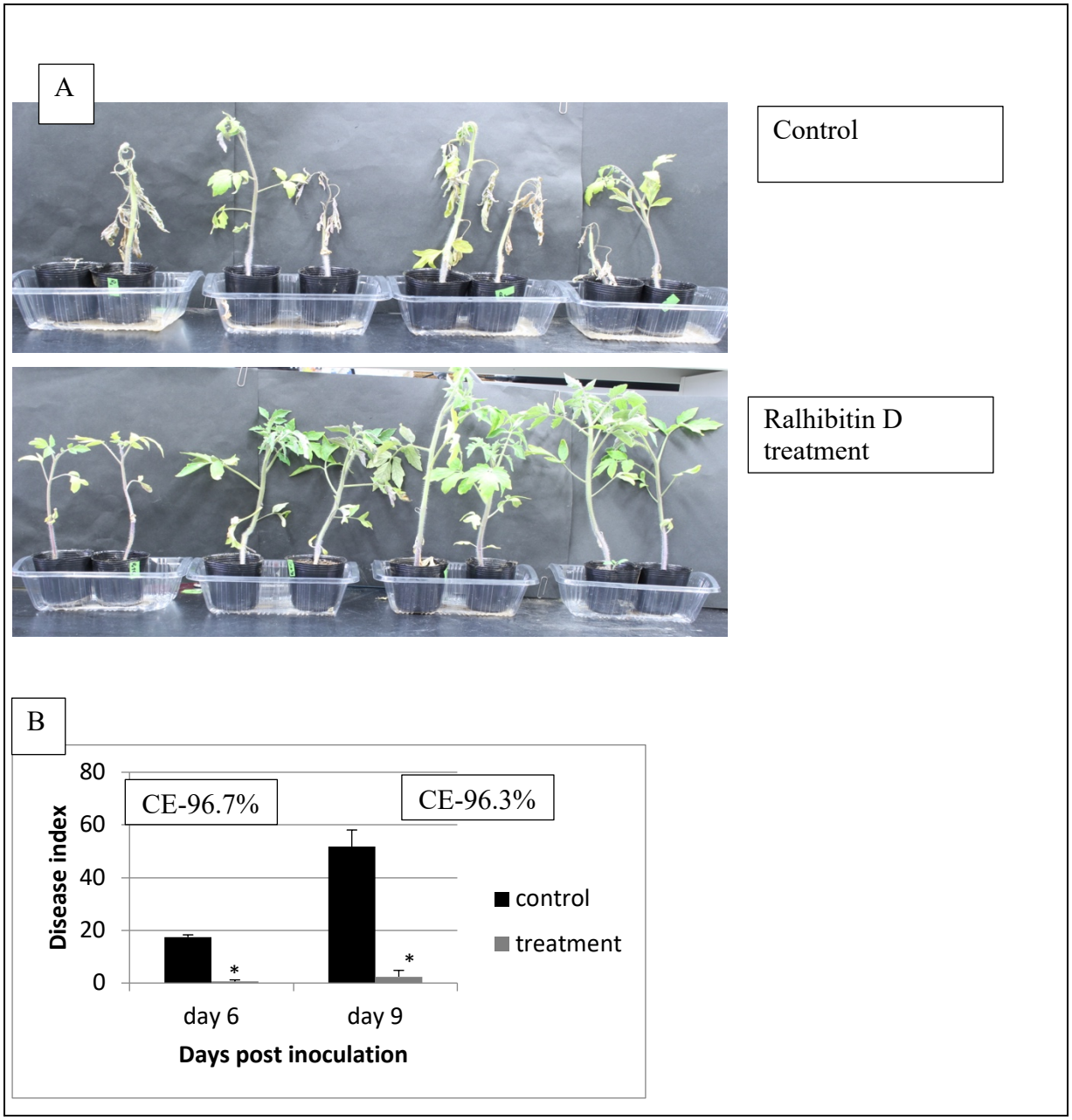

Fig. 3.1. a) Effect of 111 µg/ml of ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.06) at 10 DPI, b) Disease index and control efficiency. The disease index and control efficiency values are means of 3 independent experiments. Error bars represent standard deviations. Disease index differences at **P*<0.05 were considered significant.

For instance, at 9 DPI, control efficiency is calculated as; CE (1), (46.9-0)/46.9 ×100, CE (2), (43.8-0)/43.8×100, CE (3) (64.3-7.1)/64.3×100. Therefore at 9 DPI, control efficiency 96.3% is the average of the 3 CE values, (100+100+89)/3

Similarly, at 56 µg/ml under low *R. solanacearum* bacterial population (OD_{660nm} = 0.06), wilting symptoms characteristic of *R. solanacearum* were recorded in tomato seedlings at 6 DPI. Significant differences at ($p<0.05$) were observed between the control group of tomato seedlings and the ralhibitin D treated tomato seedlings (Fig. 3.2a). The disease index for the control group was 17.6 at 6 DPI while that of treatment was 2.1. At 10 DPI, the disease index of control group increased markedly to 54.8 while that of treatment was 6.3. The control efficiency at 56 µg/ml was 88.9% at 6 DPI and 87.7% at 10 DPI, respectively (Fig. 3.2b)

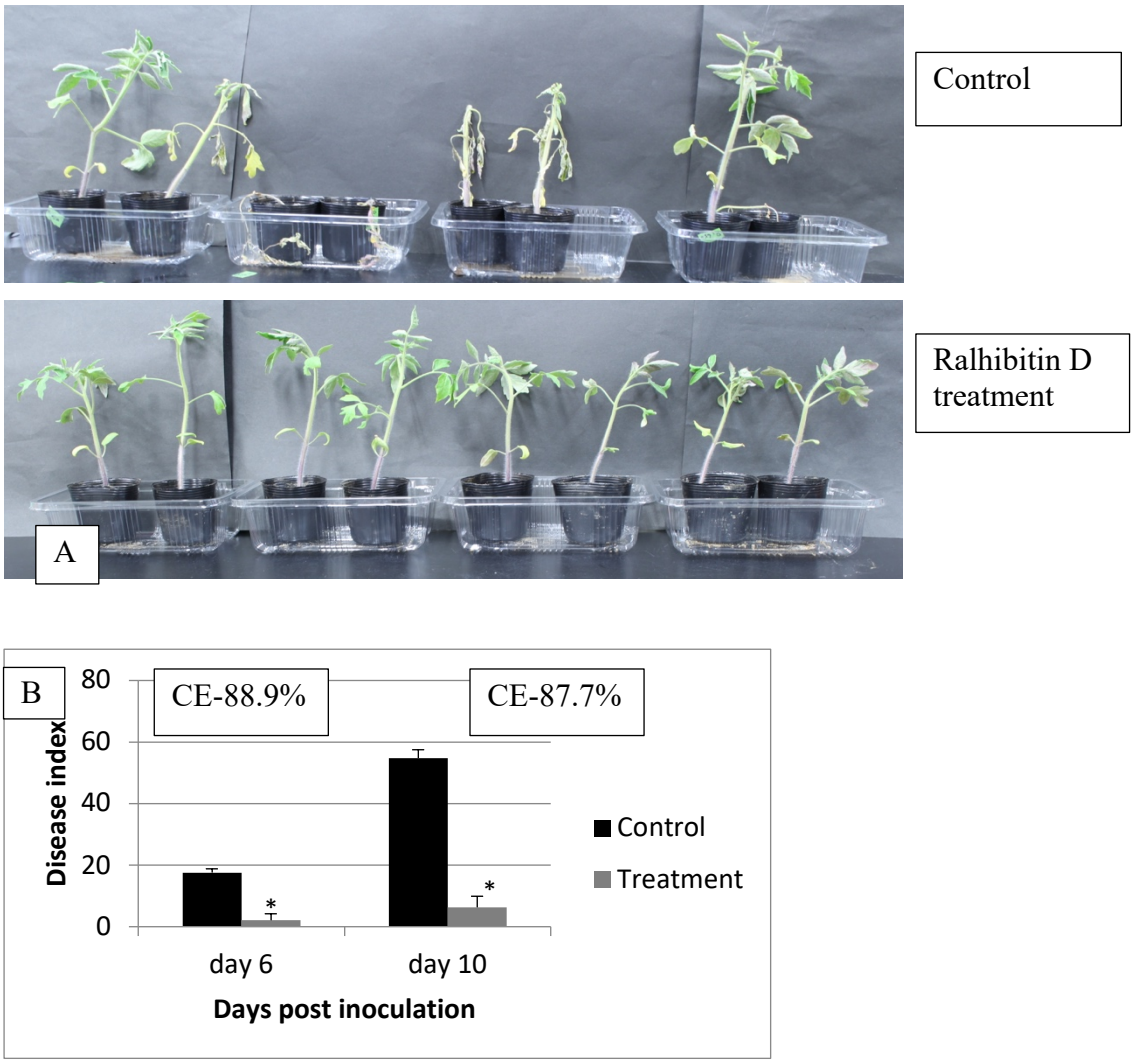

Fig. 3.2. a) Effect of 56 µg/ml ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.06) at 10 DPI, b) Disease index and control efficiency (CE). The disease index and control efficiency values are means of 3 independent experiments. Error bars represent standard deviations. Disease index differences at *$P<0.05$ were considered significant.

At 28 µg/ml, both the control group and ralhibitin-treated group of tomato seedlings had wilting symptoms at 6 DPI. There were no significant differences at $p<0.05$ between the control and treatment at this chemical concentration (Fig. 3.3a). The disease index at 6 DPI were 25.2 for control and 28.5 for treatment respectively. At 9 days post inoculation, the disease index of control was 64.8 while that of ralhibitin D treatment was 71.7 (Fig. 3.3b) There was no protective effect against *R. solanacearum* at this low chemical concentration.

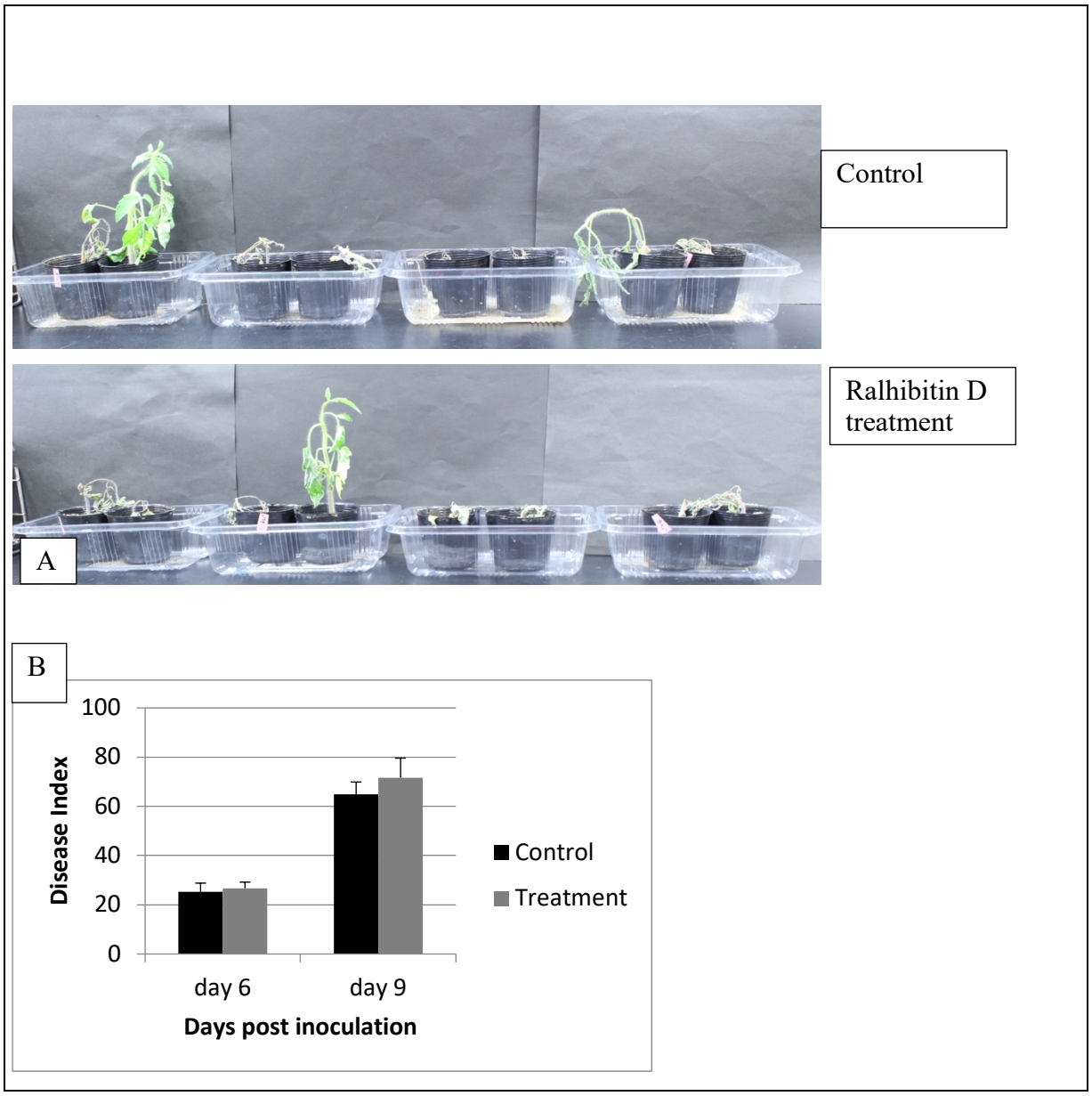

Fig. 3.3. a) Effect of 28 µg/ml ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.06) at 10 DPI, b) Disease index. The disease index and control efficiency values are means of 3 independent experiments with 8 replicates each. Error bars represent standard deviations.

Further decrease in ralhibitin D concentration to 14 µg/ml resulted in loss of protective ability as seen by wilting of plants in both the control and treatment group (Fig. 3.4a). At this concentration, there were no significant differences in disease index between control and treatment group at ($p<0.05$). The disease index of control at 6 DPI was 23 whereas that of ralhibitin D treated seedlings was 22.3. At 9 DPI, the disease index for control was 44.9 while that of treatment was 49.4 confirming the loss of protective activity against *R. solanacearum* (Fig. 3.4b)

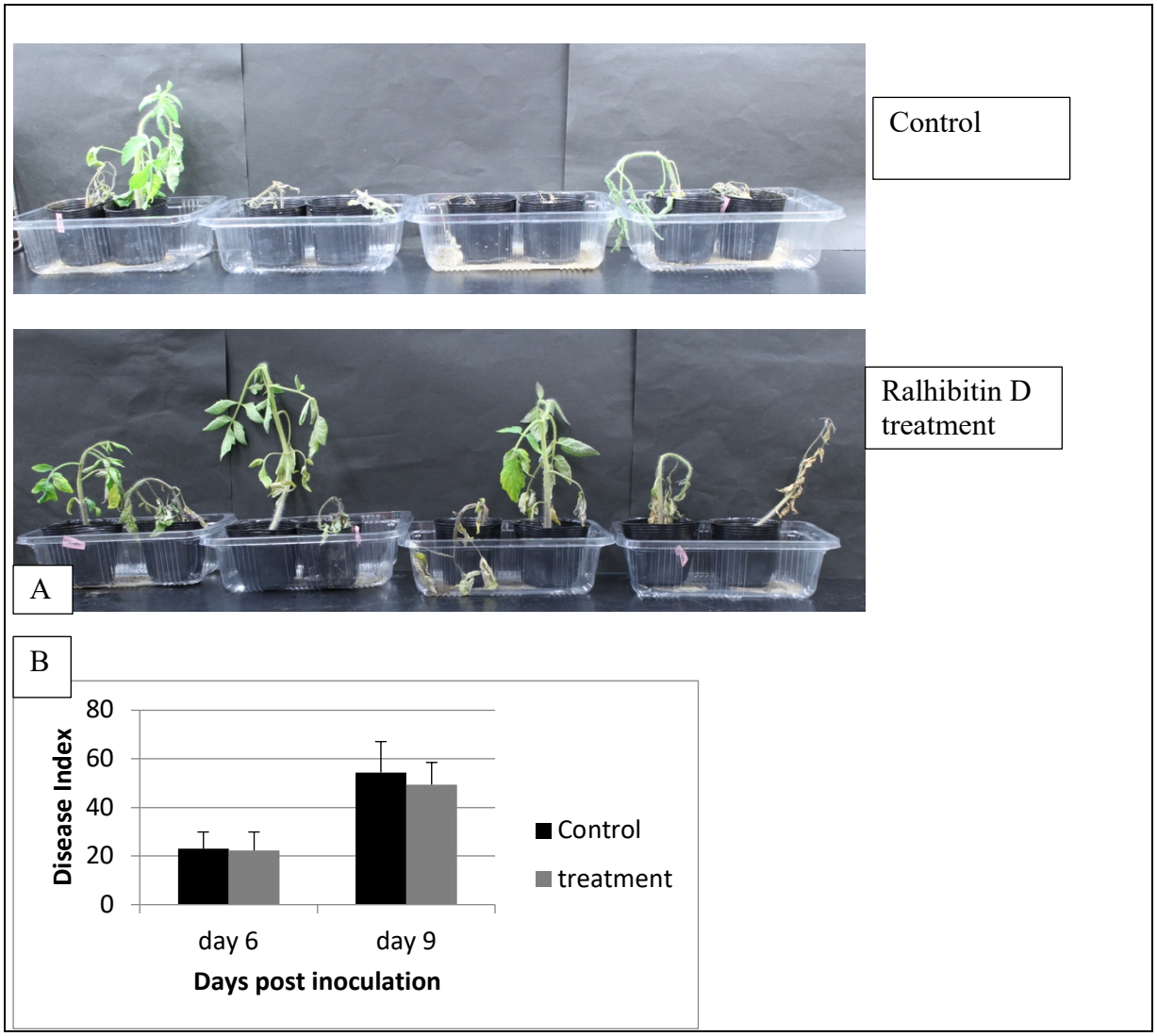

Fig. 3.4. a) Effect of 14 µg/ml ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.06) at 10 DPI, b) Disease index. The disease index and control efficiency values are means of 3 independent experiments with 8 replicates each. Error bars represent standard deviations.

2. Ralhibitin D has protective activity against tomato seedlings at higher *Ralstonia solanacearum* concentration (OD_{660nm} = 0.2)

There are varying levels of bacterial populations in the natural field conditions with some fields containing high and others low inoculum, respectively. It's therefore necessary to determine the effect of ralhibitin D on tomato seedlings inoculated with higher bacteria inoculum (OD_{660nm} = 0.2) of *R. solanacearum*. The control group were tomato seedlings inoculated with only *R. solanacearum* at higher inoculum. Treatment of tomato seedlings with 111 µg/ml ralhibitin D showed protective effect against *R. solanacearum* (Fig. 3.5a). There were significant differences between the disease index of control group and ralhibitin D-treated tomato seedlings. The disease index at 6 DPI was 27 for control group and 5.2 for ralhibitin D treatment. Remarkable increase in disease index for the control group was observed at 10 DPI recording 70.9 whereas that of ralhibitin D-treatment increased slightly to 17.7. The control efficiency at 111 µg/ml was determined to be 87.2% at 6 DPI and 75.4% at 10 DPI (Fig. 3.5b)

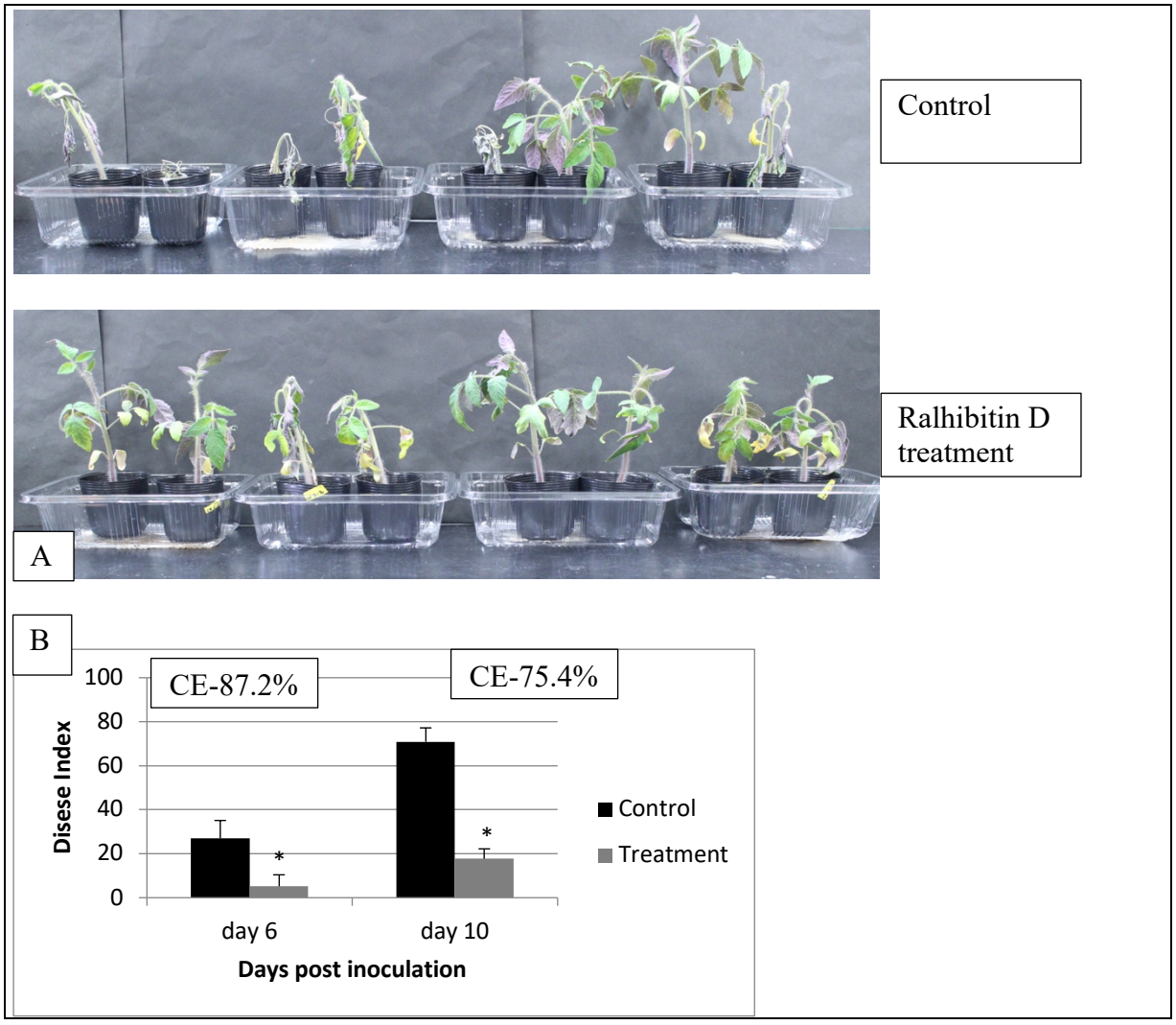

Fig. 3.5. a) Effect of 111 µg/ml ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.2) at 10 DPI, b) Disease index and control efficiency (CE). The disease index and control efficiency values are means of 3 independent experiments with 8 replicates each. Error bars represent standard deviations. Disease index differences at $*P<0.05$ were considered significant. At 10 DPI, control efficiency is calculated as; CE (1), (59.4-9.4)/59.4 ×100, CE (2), (81.3-18.8)/81.3×100, CE (3) (71.9-25)/71.9×100. Therefore at 10 DPI, control efficiency 75.4% is the average of the 3 CE values, (84.2+76.9+65.2)/3

Similarly, at lower ralhibitin D concentration of 56 µg/ml, protection against *R. solanacearum* was achieved (Fig. 3.6a). However, the protection level at this ralhibitin concentration decreased marginally. Wilting symptoms occurred first at 7 DPI for both control and ralhibitin D treated tomato seedlings. Significant differences in disease index at ($p<0.05$) was observed between the ralhibitin D-treated and control group tomato seedlings. At 7 DPI, the disease index of control was 14.5 while that of ralhibitin D-treatment was 6.9. the disease index at 10 DPI was 53.4 for the control group and 19.5 for the ralhibitin D-treated seedlings. The control efficiency at 7 DPI was determined to be 53.3 while that of ralhibitin D-treated seedlings at 10 DPI was 69.2 (Fig. 3.6b).

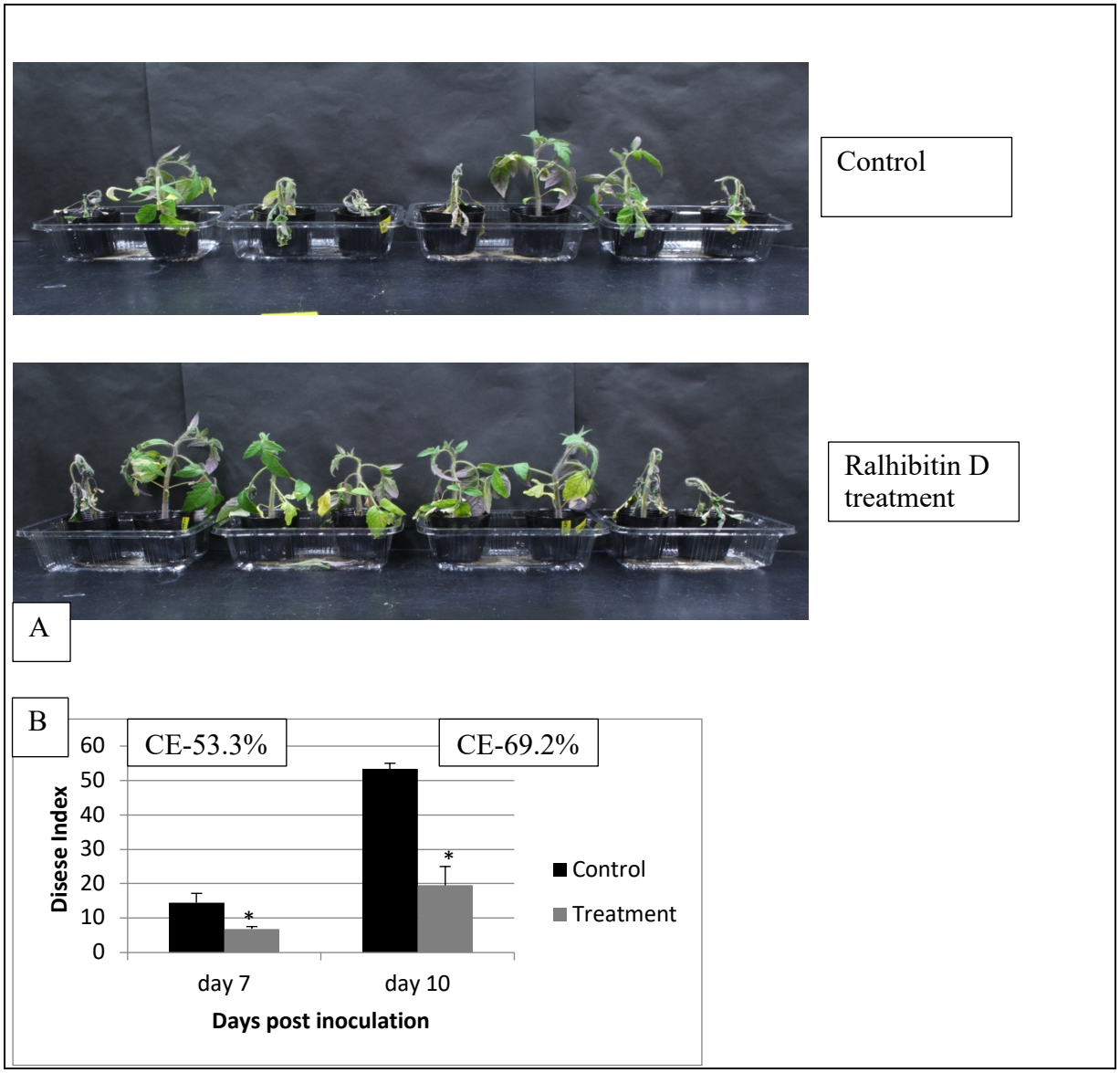

Fig. 3.6. a) Effect of 56 µg/ml ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.2) at 10 DPI, b) Disease index and control efficiency (CE). The disease index and control efficiency values are means of 3 independent experiments with 8 replicates each. Error bars represent standard deviations. Disease index differences at *P<0.05 were considered significant.

Further decrease in ralhibitin D concentration to 28 µg/ml at higher bacteria population resulted to abolishment of protective ability *R. solanacearum* (Fig 3.7a). The wilting symptoms characteristic of *R. solanacearum* were present in both control and ralhibitin D-treated seedlings and being more pronounced in the ralhibitin D-treatment at 6 DPI. There was no significant difference at $p<0.05$ between the ralhibitin D-treatment and control group of tomato

seedlings. The disease index of control group was 15.7 at 6 DPI while that of ralhibitin D-treatment was 18.7. Similarly, the disease index of control and ralhibitin D-treatment were almost similar at 10 DPI being 56.3 and 44.8, respectively (Fig. 3.7b).

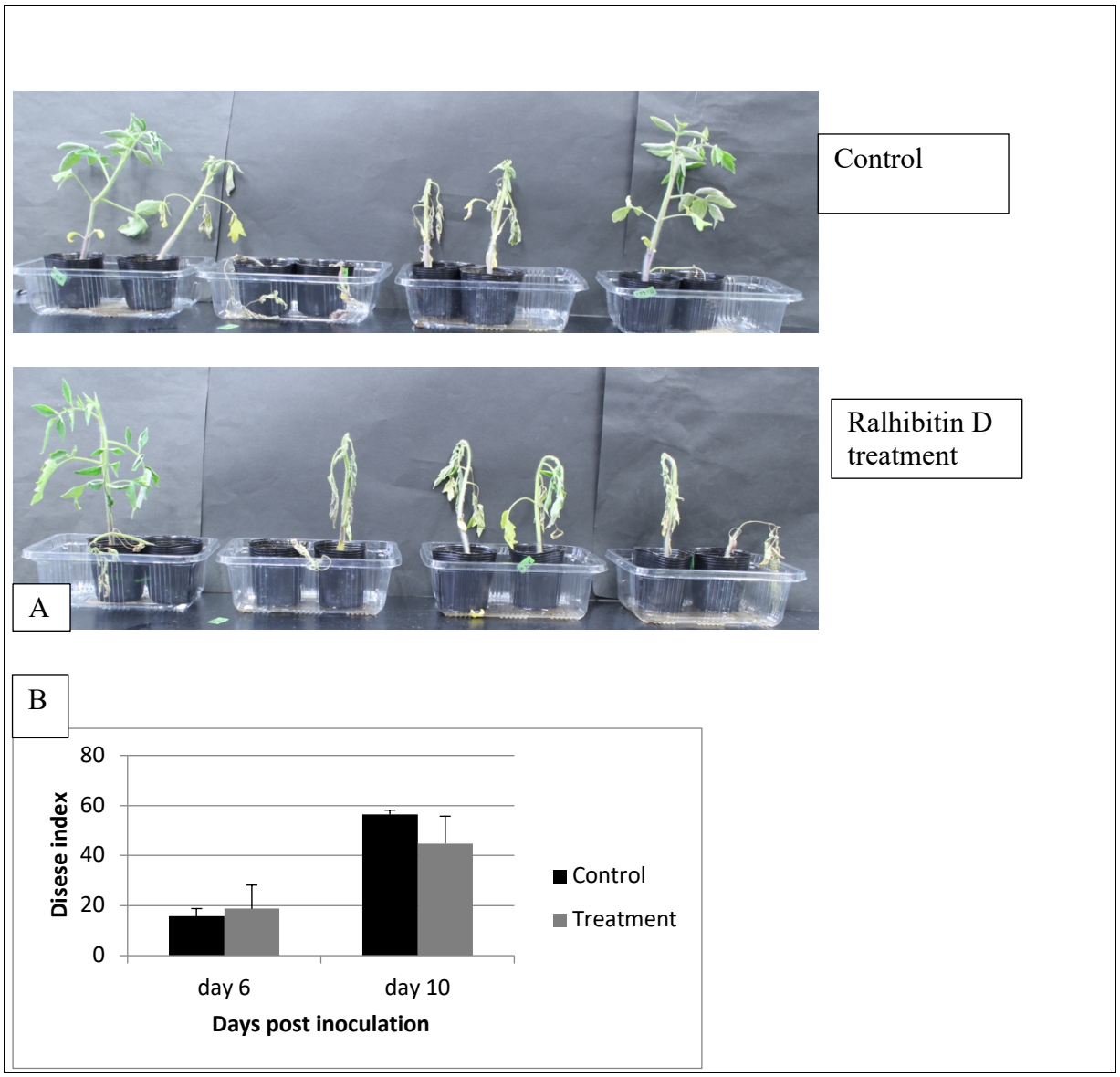

Fig. 3.7, a) Effect of 28 µg/ml ralhibitin D on *R. solanacearum* infected tomato seedlings (OD_{660nm} = 0.2) at 10 DPI, b) Disease index. The disease index and control efficiency values are means of 3 independent experiments with 8 replicates each. Error bars represent standard deviations.

IV. DISCUSSION

The pathogen *R. solanacearum* is widely distributed and is considered the 2nd most important bacterial pathogens (Mansfield et al., 2012). The study of host-bacteria interactions has utilized *R. solanacearum* as a model system (Sing et al., 2018). Control of this bacteria is limited by its

systemic nature, presence of a large host range, wide distribution and the existence a species complex (Yang et al., 2018). Many control strategies have been attempted such as breeding for resistance, chemical, cultural, integrated management and biological control (Yuliar et al., 2015). The commercial compounds that have been reported to significantly reduce or delay the severity of bacterial wilt include copper oxychloride-dithianon, copper hydroxide and copper hydroxide-oxadixyl (Lee et al., 2012). These compounds were reported to have been applied concurrently with *R. solanacearum* infection (Hong et al., 2013). Some integrated disease management options against *R. solanacearum* have also been reported such as the use of rhizobacteria, essential oils and bio-control agents (Anith et al., 2004; Ji et al., 2005; Nakaune et al., 2012; Park et al., 2007). The traditionally utilized chemical compounds such as thiadizole copper and streptomycin have shown limited efficacy in field conditions (Yang et al., 2018). Similarly, it has been reported that there is low acceptability of resistant cultivars by consumers and farmers due to some undesirable agronomic traits they possess (Yuliar et al., 2015). Because of the limitations of the current control methods, research for newer and alternate control methods is ongoing.

My previous *in vitro* results showed that ralhibitins had growth inhibitory effect against *R. solanacearum* at low concentrations vitro (Below 10 μg/ml) (Ombiro et al., 2018). The growth inhibition of pathogens at concentrations below 10 μg/ml is considered interesting and with potential for further development (Babii et al., 2018). This results are also better than those reported for copper nanoparticles against *R. solanacearum* where the inhibitory effect was achieved at 250 μg/ml (Chen et al., 2019). The ralhibitins A-E obtained in our previous study Ombiro et al. (2018) are therefore interesting and promising. Due to these promising results, the activity of ralhibitin D against *R. solanacearum* in *vivo* at different concentrations was determined in pot experiments. Our study used tomato as a test plant due to its preference as a model when the pathogen under study is soil borne (Sing et al., 2018). We utilized soil-drenching method because it can mimic the natural infection method of soil borne pathogens such as *R. solanacearum*. The pattern and behavior of the pathogen after introduction by other methods such as stem inoculation may be significantly different. This system has also been reported to be effective in studying pathogenicity confirming the importance of the method (Singh et al., 2018).

In the current study, I determined that ralhibitin D could protect tomato seedlings from *R. solanacearum* infection at a lower bacterial concentration. The suppression effect was dose dependent. Control efficiency of 88.7 was also observed at 10 DPI after treatment with 56

µg/ml. These results are better than those reported by Yang et al. (2018) on the effects of some hydroxycoumarins namely; umbelliferone, daphnetin and esculetin on bacterial wilt in tobacco. Daphnetin (DA) significantly suppressed the disease with control efficiency of 59.2 at 10 DPI (Yang et al., 2018). This shows that ralhibitin D is more effective against *R. solanacearum* as compared to daphnetin. Another study investigated the effect of lansiumamide B on tobacco bacterial wilt reporting control efficiency of 91.7 at 14 days post inoculation (Li et al., 2014). Another study has also reported efficiency of resveratrol against *R. solanacearum* with a reported control efficiency of 68% at 13 days post inoculation (Chen et al., 2016).

Some plant derived tannin compounds have also been shown to have inhibitory effect against *R. solanacearum* but only at higher concentration of 1000 µg/mL (Vu et al., 2017b). After 14 days, these compounds had control efficiency of 63. However, when the concentration of the extract was increased further to 2000 µg/mL the efficiency also increased to 83 similar to the dose dependent activity of ralhibitin D in my study. The control efficiency of 65.2 has been reported for methyl gallate at a concentration of 500 µg/mL against *R. solanacearum* in the greenhouse studies (Vu et al., 2017b). Some metabolites from pharbitin seeds have also been reported to have control efficiency of 97.4 at 7 days post inoculation at a higher concentration of 250 µg/ml (Nguyen et al., 2017).

Similarly, protection effect was achieved in tomato seedlings inoculated with higher bacteria inoculum. The greatest effect was observed after treatment with 111 µg/ml ralhibitin D that resulted in a significant difference of disease index being 17.7 at 10 days post inoculation for ralhibitin D treatment as compared to that of control at 70.9. These results suggest that at higher bacterial populations, higher chemical concentration are needed to enhance protection. These results are in agreement with a study using perosan, which reported the need for higher perosan amounts (0.0006%) to enhance protection at higher bacterial populations (10 cfu/ml). A similar effect has been reported for peroxyacetic acid mixture (composed of acetic acid, hydrogen peroxide and peroxyacetic acid) that can control *R. solanacearum*. There was delayed protective effect when 0.01% of the mixture was applied on detached leaves (Hong et al., 2018). On the other hand, drenching of 1% of the mixture in pots with tomato seedlings had a significant effect on bacteria wilt severity.

V. EXPERIMENTAL PROCEDURE

1. Ralhibitin D source and preparation

Ralhibitin D was purchased from Alinda chemicals in powder form and stored in -20°C freezer. Previously assays were conducted using ralhibitins dissolved in DMSO but was shown to have phytotoxic effects on tomato plants. We then checked dissolution of ralhibitins in different solvents and determined that ralhibitin D could be dissolve in ethanol. Ethanol did not show phytotoxic effects on tomato plants. Ralhibitin D was prepared by dissolving 10 mg in 1 ml ethanol and this formed the stock solution. The stock solution was also kept at -20°C and constituted for application as necessary.

2. BG medium preparation

BG medium was prepared by the ingredients (Bacto peptone 10 g, yeast extract 1 g, casamino acid 1 g/L water, pH 7). Briefly, the ingredients were weighed, placed into a beaker then 1000 ml of distilled water was added and stirred until complete dissolution and pH 7. Then 100 ml of the solution was aliquoted into conical flasks with and without 1.5 grams of agar and autoclaved at 121°C. The agar containing medium was then utilized to prepare BG plates.

3. Growth of tomato seedlings

Tomato seeds of cultivar Ponderosa were grown in sterilized vinyl plastic pots with a volume of 100 mL for one week. Firstly, soil was filled into the vinyl pots up to 80 ml volume and then the pots were watered until run off. Seeds were then placed on the wet soil medium and covered. The planted seeds were watered regularly by use of sterile distilled water until they germinated. The plants were maintained in a growth chamber with controlled conditions with temperature at 28°C. Then they were transplanted to individual pots and grown for further two weeks. Seedlings of the same height were then selected for infection assay.

4. *R. solanacearum* inoculum preparation

*R. solanacearum Rs*1002 obtained from a glycerol stock at -80° C was streaked onto BG (Bacto peptone 10g, Yeast extract 1 g, casamino acid 1 g) agar plates and incubated overnight at 27°C.

Then colonies were added into 50 ml BG medium liquid culture by a sterilized wire loop and incubated at 27°C shaking at 200 rpm overnight for 24 hours. The *R. solanacearum* overnight culture was centrifuged at 6000 rpm for 5 minutes and supernatant was discarded. Then distilled water was used for resuspension of the pellet to OD_{660nm} = 0.06 and 0.2, respectively. Next, 1300 µl, 650 µl, 325 µl and 163 µl from a stock of 10 mg/mL of Ralhibitin D in ethanol was added to 90 ml of water before addition of 25 ml of bacteria suspension and briefly mixing making a final concentration of 111, 56, 28 and 14 µg/mL, respectively.

5. Inoculation of tomato seedlings with *R. solanacearum* of OD_{660nm} = 0.06

Uniform 3-week-old tomato seedlings in individual plastic vinyl pots were obtained from growth chamber and used for *in planta* protection assay. Prior to inoculation, the moisture content of the pots was minimized by reduced watering to avoid run off of inoculum. Each treatment had eight plants in every independent experiment and the protection assay was repeated three times. The control seedlings were inoculated with the pathogen without ralhibitin D. To the selected individual plants, 13 ml of bacteria chemical suspension at bacteria OD_{660nm} 0.06 was drenched to the roots. The plants were incubated in growth chamber under 16 h light/8 h darkness cycle at 28°C and 80% humidity. Then symptoms were scored daily for 9-10 days using a severity scale of (0-4) as described by Zheng et al. (2014): 0, healthy plant and 4, dead plant. Further to this, dose dependent protection assay was done. To obtain, 56 µg/ml final concentration, 650 µl of ralhibitin D was added to 90 ml of water before the addition of 25 ml of *R. solanacearum* of OD_{660nm} 0.06 . Then 13 ml was drenched to the roots of tomato seedlings. Addition of 325 µl and 163 µl to 90 ml water provided a final concentration of 28 and 14 µg/ml respectively. Then 13 ml of this solution was drenched to the roots of tomato seedlings and symptom severity was recorded. The experiments were carried out using 8 tomato plants for each treatment and repeated three times in a complete random block design.

6. Inoculation of tomato seedlings with *R. solanacearum* of OD_{660nm} = 0.2

Secondly, tomato seeds were sown on sterilized soil medium in 100 ml pots. Three weeks old seedlings were used for protection assay. An overnight culture of *R. solanacearum* in 3 ml BG medium was prepared. Then the culture was centrifuged at 6000 rpm for 5 minutes and the absorbance adjusted to OD_{660nm} = 0.2 by addition of water to the bacterial suspension. Next, 1300 µl of Ralhibitin D was added to 90 ml of water before addition of 25 ml of bacteria suspension and briefly mixing to make final concentration of 111 µg/ml. To three weeks old

seedlings, thirteen ml of bacteria plus chemical suspension was drenched to the roots of three weeks old seedlings. Then the plants were incubated in the growth chamber at constant temperature and humidity. Then symptoms were scored using a severity scale of 0-4. Further to this, dose dependent protection assay was done. To obtain the disease index at 56 µg/ml ralhibitin D of final concentration, 650 µl of ralhibitin D (56 µg/ml)was added to 90 ml of water before the addition of 25 ml of *R. solanacearum* OD_{660nm} 0.2. Then 13 ml was drenched to the roots of tomato seedlings. Addition of 325 µl to 90 ml water provided a final concentration of 28 µg/ml. Then 13 ml of this solution was drenched to the roots of tomato seedlings and symptom severity was recorded at 6 and 10 days post inoculation.

7. *R. solanacearum* symptom assessment

Disease occurrence was assessed through visual observation for wilted plants using a severity scale (Fig. 3.8). *R. solanacearum* disease symptoms such as wilting of leaves and entire plant were evaluated daily using disease severity scale of 0-4 with rating score in Table 3.1 (Zheng et al., 2014). Measurements were done on appearance of the first wilting symptoms until 10 days post inoculation.

Table 3.1: Disease rating and corresponding symptom expression for *R. solanacearum* (Zheng et al., 2014).

	Rating
0	Asymptomatic (healthy plant)
1	One fourth of the whole leaves were wilted
2	Half of the whole leaves were wilted
3	Two thirds of the whole leaves were wilted
4	The entire plant died

Fig 3.8. Infected tomato plants showing varying severity scale of bacteria wilt. Severity frequency was calculated (Yang et al., 2018). Disease index = Σ (ni× vi) ×100/N×4.

Where ni -number of plants with particular disease index, and vi, the respective disease index (0, 1, 2, 3,4). N is the cumulative number of plants in each treatment. Control efficiency = 100(C-T)/C. Here, T represents the treatment disease index whereas C represents disease index of control group.

8. Statistical analysis

The results of *R. solanacearum* symptom severity were first converted to disease index, then converted to control efficacy. They disease index values were then analyzed by ANOVA through comparisons between treatment disease index and control disease index. The values ($P<0.05$) were considered significant.

CHAPTER IV

Identification of Ralhibitins Target Site by Transposon Mutagenesis

I. SUMMARY

Generation of genetic insertions libraries in many bacteria has been accomplished by random transposon mutagenesis. I identified compounds (ralhibitins) that can inhibit the growth of some plant pathogenic bacteria *Rs, Xoo, Xcc* and *Cmm*. Using random transposon mutagenesis, I attempted to identify the genes targets for ralhibitins in *R. solanacearum*. I obtained many chemical resistant transconjugants in *R. solanacearum* by conjugation. Using the plasmid rescue procedure, I obtained sequences flanking the transposon insertion site and then mapped them to the genome of *R. solanacearum*. The transposon was inserted randomly to multiple genes that do not have a role in *R. solanacearum* ralhibitin susceptibility. Also, in my study, most bacteria are tolerant to ralhibitins, and only limited bacteria including *R. solanacearum* are sensitive to ralhibitins. However, the sensitivity mechanism of *R. solanacearum* to ralhibitins is still not clear yet. To determine the mechanism how ralhibitins inhibit the growth of *R. solanacearum*, I investigated the effect of spent culture medium of insensitive bacteria with ralhibitins on the growth of *R. solanacearum* (*Rs*1002) and found that spent cultures did not inhibit the growth of *R. solanacearum*. This result indicated that the ralhibitin-insensitive bacteria may be able to inactivate the inhibitory effect of ralhibitins. Therefore, I conducted a transposon mutagenesis experiment that may identify the genes related to ralhibitin-tolerance using ralhibitin-insensitive bacterium *Pseudomonas syringae* pv. *tomato* DC3000. In this screening, the transposon miniTn5 was randomly inserted into the genome of *P. syringae* pv. *tomato* DC3000 by conjugation, and ralhibitin-sensitive transconjugants were selected. However, strong candidates for the genes involved in tolerance to ralhibitins were not obtained yet.

II. INTRODUCTION

Bacterial inhibitors and antibiotics target different bacterial sites to inhibit growth. The macrolide antibiotics affect protein synthesis through inhibition of conserved sequences of the 23S rRNA leading to detachment of peptide chains prematurely (Wise, 1989). A compound known as auranofin that inhibits Gram-negative bacteria has been reported to act through inhibition of biosynthetic pathways such as DNA, bacterial protein synthesis and the cell wall (Thangamani et al., 2016). On the other hand, a broad spectrum compound rifampicin acts through inhibiting the bacterial DNA-dependent RNA polymerase (Campbell et al., 2001). Some antimicrobial peptides have been shown to act by inhibiting the cell wall synthesis through disruption of cell wall synthesis precursor molecules (Kumar et al., 2018). The conserved lipid II is one of the precursor molecules targeted by the defensin peptides through

the binding of negatively charged sugar moiety. The mode of action of zinc nanoparticles that have antibacterial activity against *E. coli* has been reported to be through membrane disorientation increasing permeability and subsequent nanoparticles accumulation in the bacterial cell (Raghupathi et al., 2011).

On the other hand, tolerance to antibiotic compounds is through different mechanisms employed by the target bacteria. Some enzymes; chloramphenicol acetyltransferases, beta-lactamases have been reported to be involved inactivation of antibiotics (Dockrell et al., 2004). For instance, cephalosporins and penicillin are known to be inactivated by beta-lactamases (Alekshun and Levy, 2007). Other tolerance mechanism includes the target molecule modification through mutations of genes in a bacterium therefore affecting binding of antibiotics. For example, chloramphenicol transacetylase makes the antibiotic fail to bind to the ribosomal 50S subunit and disable inhibitory effect (Tolmasky, 2000). Another mechanism for resistance to antibiotics has been through efflux pumps. This involves the removal antibiotics from the bacteria before arrival at their target (Wise, 1999). In my previous study (Ombiro et al., 2018), I identified compounds with inhibitory effect against some Gram-negative bacteria (*Rs, Xcc, Xoo*) and Gram-positive bacteria *(Cmm)*. Also, in the same study, many phytopathogenic bacteria were found to be tolerant to the ralhibitins. Unravelling the mode of action for susceptibility and tolerance to ralhibitins is therefore important.

III. RESULTS

1. Identification of genes involved in ralhibitin susceptibility in *R. solanacearum*

1.1. Screening for ralhibtin A resistant transconjugants in *R. solanacearum*

Screening of chemical compounds against *R. solanacearum* determined that ralhibitin A could inhibit its growth at a final concentration of 10 µg/ml. This final concentration was then used to screen for tolerant mutants from a Tn5 transposon library of *R. solanacearum* strain RS1002. To identify genes involved in suceptibility of *R. solanacearum* to ralhibitins, a transposon library of *R. solanacearum* (recipient) was created through conjugation with *E.coli* S17-1 containing plasmid pBSLC1 (donor), a kanamycin resistant mini-Tn5 transpson vector (Fig. 4.1).

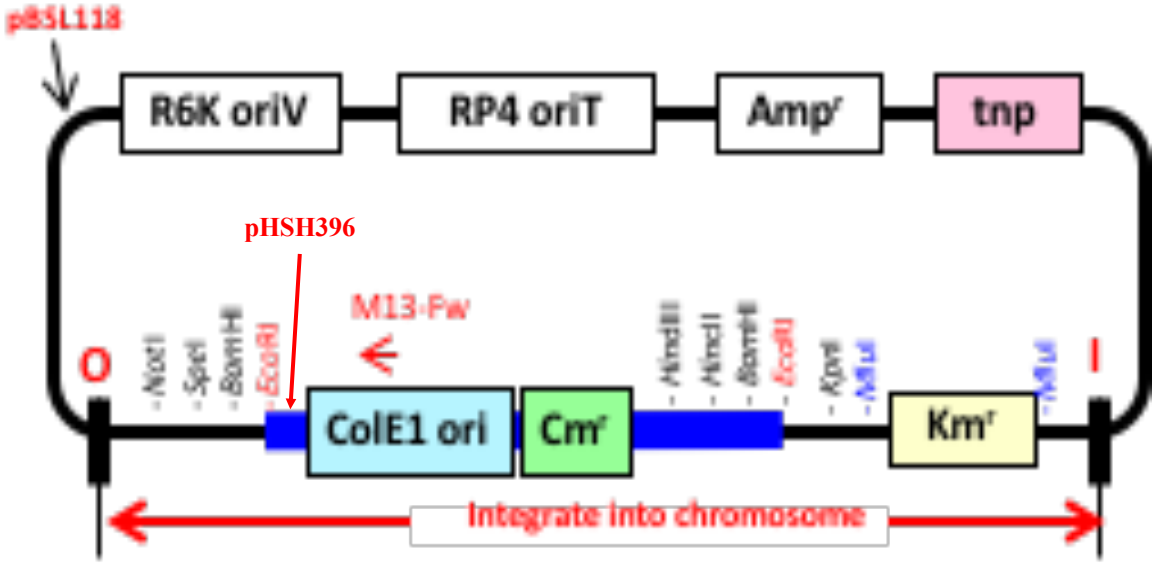

Fig. 4.1. Transposon vector pBSLC1 used for transposon mutagenesis through conjugation with RS1002. It contains, R6K *oriV*, origin for DNA replication in *E.coli*, RP4 *oriT*, origin for intergeneric conjugation from *E. coli* to *Ralstonia solanacearum*, *tnp* encoding a transposase, *Km*^{r}, kanamycin resistance marker.

Colonies growing on BG plates containing antibiotics markers (Nalixidic acid and Kanamycin) are referred to as transconjugants. Nalixidic acid selects for RS1002 whereas kanamycin selects for insertion of the transposon vector pBSLC1. Further screening was done to select for transconjugants that can grow on BG, Kanamycin, Nalixidic acid plus ralhibitin A (10 µg/mL). Colonies that grew on ralhibitin plate were considered as ralhibitin A tolerant mutants. Many colonies were obtained from this screening after growing on plates containing ralhibitin A. The ratio of chemical (ralhibitin A) transconjugants to non-chemical transconjugants was 1:2 (Fig. 4.2).

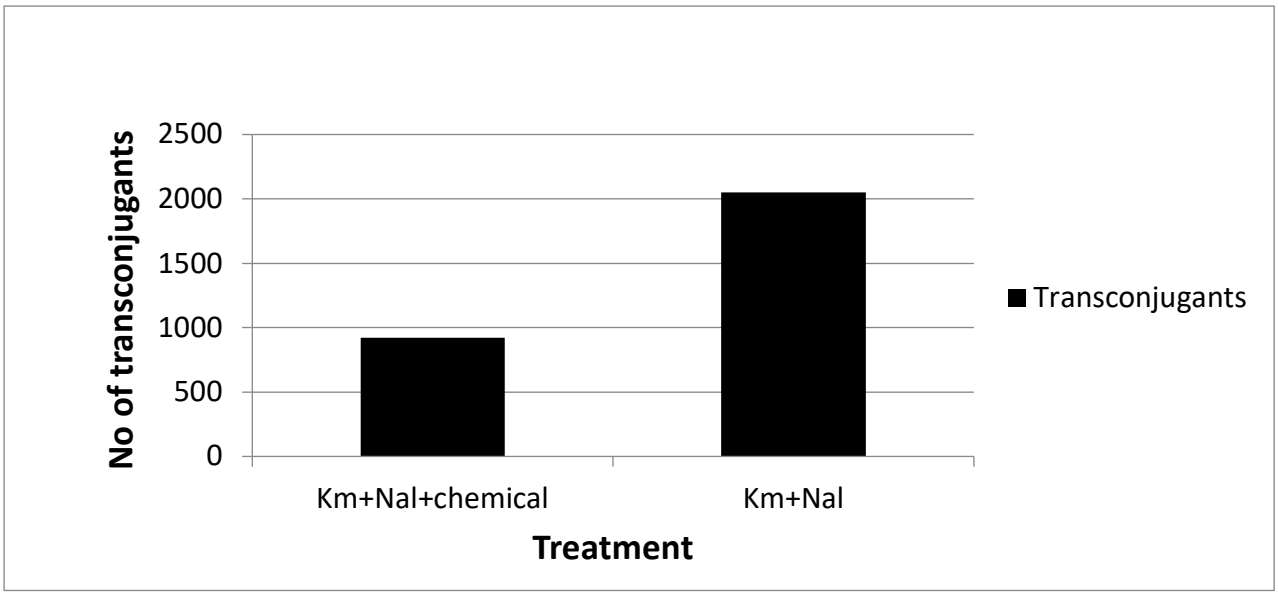

Fig. 4.2, Transconjugants obtained after conjugation between *R. solanacearum* and *E. coli* S17-1 (pBSLC1).

1.2. Identification of disrupted genes by plasmid rescue

Plasmid rescue is involved in retrieval of insertion region flanking sequence through total genomic DNA digestion from mutants by enzymes not cutting within the transposon, followed by self-ligation and subsequent transformation into *Eschelichia coli* (competent cell). In my study, the region (IO) in transposon vector pBSLC1 was integrated into the genome of RS1002 randomly causing disruptions to different genes. Screening of the transconjugants on ralhibitin A obtained several colonies that were tolerant. Genomic DNA was successfully isolated from the tolerant transconjugants followed by digestion with an enzyme that does not cut within the transposon vector pBSLC1 (In this case *Hin*dIII) followed by ligation and transformation into a competent cell DH5α. Plasmid isolation from obtained colonies and sequencing through outward facing primer (M13 F primer) obtained the sequence flanking the transposon insertion site.

1.3. Blast search to identify proteins homologous to sequences flanking transposon insertion point

Sequences obtained from flanking regions of the transposon were mapped on to the genome of *R. solanacearum*. By using the BLASTP algorithm, the obtained sequences were matched to

protein sequences in the NCBI for homology. The results however indicated that the transposon was inserted in many genes within the genome of *R. solanacearum* which have no role in ralhibitin susceptibility. This meant that I was not able to identify candidate genes involved in ralhibitin susceptibility in pathogen *R. solanacearum.* I hypothesized that the presence of the chloramphenicol gene (Cm^r) in the transposon vector pBSLC1 could have a role in detoxification of ralhibitin A.

2. Identification of genes involved in tolerance to ralhibitins

2.1 Mode of action of ralhibitins

The mode of action of the ralhibitins identified in this study has not been elucidated. I determined that many bacteria are tolerant to ralhibitins such as *Bulkhoderia glumae* (*Bg*), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Rhizobacteria radiobacter* (*Rr*). To try and understand their working mechanism, I examined whether ralhibitins cultured with different bacteria (*Bg, Pcc,* and *Rr*) still retained the capacity to inhibit growth of *Rs*1002. I found that RS1002 was able to grow to the control level in all culture media of *Bg, Pcc*, and *Rr* that contained ralhibitins (Fig. 4.3). Results showed that the greatest growth was in pathogen *Rr*, whereas *Bg* and *Pcc* had almost similar growth.

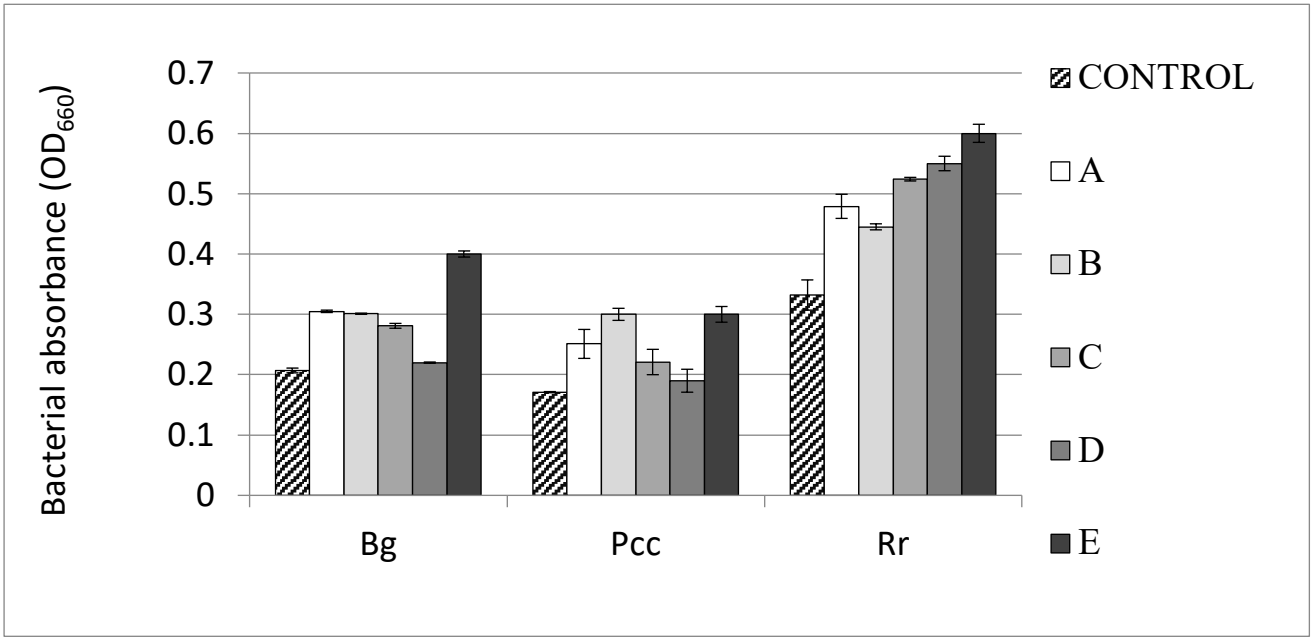

Fig. 4.3. Inhibitory effect of pre-culture with ralhibitins. Three bacteria (*Pcc*, *Bg*, and *Rr*) were incubated with 10 µg/ml ralhibitins overnight. Then the filtrate of each culture supernatant was used for the medium of *Rs*1002. Controls were precultured medium without ralhibitins. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations.

2.2 pBSG2 transposon construction

In my previous transposon mutagenesis study with *R. solanacearum*, I used transposon vector pBSLC1 for conjugation. However, this system was not successful in identifying candidate genes involved in susceptibility of *R. solanacearum* to ralhibitin. This might be probably due to the presence of a chloramphenicol resistant gene within the transposon pBSLC1 which could detoxify the ralhibitins. I then constructed a new pBSLC1 derivative named pBSG2 through deletion of the kanamycin gene in pBSLC1. This was then followed by replacement of the fragment containing the chloramphenicol resistant gene in pBSLC1 with another fragment from vector pHSG298 (Fig. 4.4). This resulted in a new transposon vector pBSG2, lacking the chloramphenicol resistant gene that was used for subsequent conjugation studies.

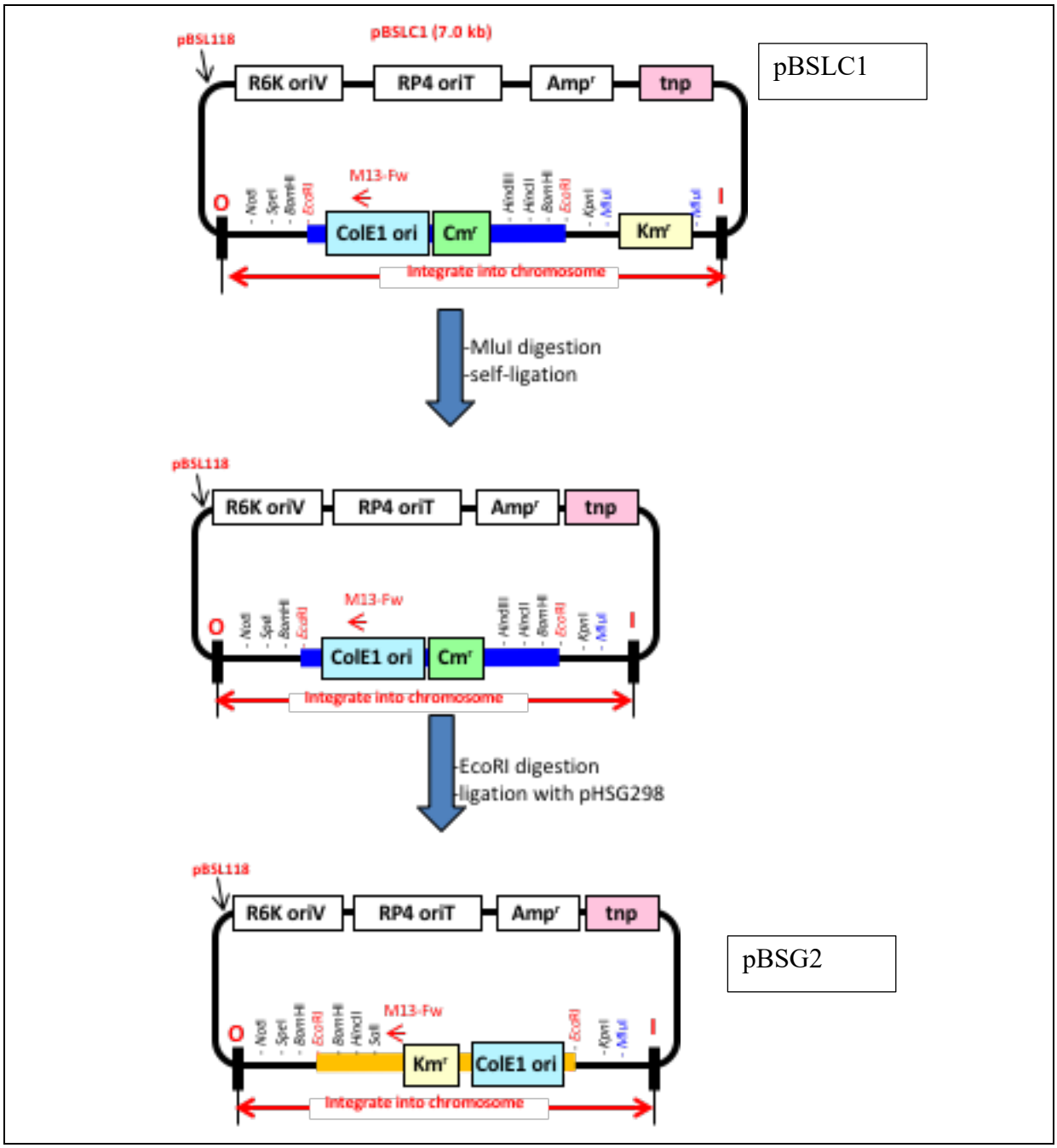

Fig. 4.4. Construction of transposon vector pBSG2

2.3. Screening for ralhibitin A sensitive transconjugants in *P. syringae* pv. tomato DC3000

Many bacteria including *Pseudomonas syringae* pv. tomato DC 3000 were found to be tolerant to ralhibitins (Ombiro et al., 2018). An assay confirmed that *Rs*1002 could grow in spent culture medium of ralhibitin tolerant bacteria, implying that the tolerant bacteria have a mechanism to detoxify or inactivate the ralhibitins. To further understand this tolerance mechanism, I

conducted a random transposon mutagenesis between *E. coli* S17-1 containing transposon vector pBSG2 (pBSLC1 derivative) as donor and pathogen *P. syringae* pv. tomato DC3000 as recipient. The transconjugants were selected on KB medium containing antibiotics nalixidic acid (Nal) and kanamycin (Km). A total of 4776 transconjugants were screened through replica plating on plates containing Nal, Km (control) or Nal, Km and ralhibitin A at 10 µg/ml (treatment). Colonies that were sensitive to the ralhibitin A were considered successful candidates containing gene responsible for the tolerant phenotype. Only one colony was found to be sensitive in my screening from a total of 4776 transconjugants.

2.4. Plasmid rescue to obtain sequences flanking the transposon insertion site in *P. syringae pv. tomato Pto* DC3000

Using restriction enzymes (*Spe1* and *Mlu1*) that do not cut inside the transposon vector pBSG2 digestion of genomic DNA of the sensitive colony was done followed by ligation and transformation. However, I could not successfully obtain plasmids containing the transposon target site and flanking region.

III. DISCUSSION

Compounds that inhibit the growth of *R. solanacearum* referred to as ralhibitins have been identified (Ombiro et al., 2018). However, their mode of action has not been unraveled, which was a subject of my investigation. Gene disruption can be done through transposon mutagenesis (Freed, 2017). In this study I successfully randomly introduced a transposon into the genome of *R. solanacearum* by conjugation and screened for transconjugants using resistant markers. Conjugation has been reported to be a cost effective and simple procedure to create large transposon mutagenesis libraries (Freed, 2017). Screening of the resultant transconjugants on ralhibitin A plates identified many tolerant colonies. Through plasmid rescue, I obtained the sequences adjacent to the transposon insertion site, and subsequently mapped them to the genome of *R. solanacearum* using whole genome sequence of the strain GM1000. The transposon insertion points were located at diverse identifiable locations within the genome of *R. solanacearum.* However, homology search using these sequences could not provide reliable candidate genes involved in *R. solanacearum* ralhibitins susceptibility. I hypothesized that the chloramphenicol resistant gene within the transposon could have had a role in detoxifying ralhibitin A. This is due to the reported role of a bacterial enzyme chloramphenicol acetyltransferase in chloramphenicol detoxification through stoppage of

ribosome binding (Dinos et al., 2016). This led to the development of a new transposon vector (pBSG2) lacking the chloramphenicol resistant gene.

Ralhibitins could inhibit the growth of some select Gram-negative and Gram-positive bacteria. However, many more phytopathogenic and a few non-plant bacteria investigated in my study were found to be tolerant to the ralhibitins including pathogen *P. syringae* pv. *tomato* DC3000. Resistance to antibiotics has been linked to a number of causes, such as the failure to penetrate the outer membrane of Gram-negative bacteria, modification of their targets, efflux of the compound from the cell or destruction of the antibiotics by enzymes (Wright, 2005). My study showed that *R. solanacearum* can grow in the spent culture medium of ralhibitin-insensitive bacteria such as *Bulkhoderia glumae, Pectobacterium carotovorum* subsp. *carotovorum* and *Rhizobacteria radiobacter* with ralhibitin. This implies that these bacteria could inactivate the inhibitory effect or detoxify ralhibitins. To understand the mechanism of detoxification, I did transposon mutagenesis using *P. syringae* pv. *tomato* DC3000. However, strong candidates for the genes involved in tolerance to ralhibitins have not been obtained.

IV. MATERIALS AND METHODS

1. Bacterial strains

Escherichia coli were grown in Luria-Bertani (LB) medium at 37°C. *Ralstonia solanacearum* was routinely grown in BG medium at 27°C. *P. syringae* pv. *tomato Pto* DC3000 was grown in KB medium. Bacteria were grown in 3 ml liquid medium in an incubator with shaking at 180 rpm 27°C. Solid medium was prepared by adding 1.5% (wt/vol) of agar. Whenever necessary antibiotics; Kanamycin (Km) (50 µg/mL), Nalixidic acid (Nal) (50 µg/mL) were used to select transconjugants containing the transposon.

2.Transposon mutagenesis

The plasmid pBSLC1/pBSG2 carrying transposon Tn5 (Km^r) in *E. coli* S17-1 was utilized to transfer the transposon into *R. solanacearum and P. syringae* DC3000, respectively. Briefly, *E. coli* S17-1 was grown overnight in 3 ml LB medium in incubator with shaking at 180 rpm, 27°C, while *R. solanacearum* or *P. syringae* pv. *tomato* DC3000 were grown in 3 ml BG or KB medium in an incubator shaking at 180 rpm, 27°C, respectively. Then, 1 ml overnight culture of each bacteria was centrifuged at 2700 rpm for 5 minutes and then 500 µL of BG or KB medium was added to the pellet. The donor and recipient cells were then mixed and

centrifuged again at 2700 rpm for 5 minutes. The obtained pellet was resuspended in 60 µL of fresh BG or KB medium. The suspension was then spotted on two nitrocellulose membrane placed on BG or KB plate without antibiotics, air-dried and incubated overnight at 27°C. Genomic DNA was extracted from transconjugants from overnight-cultured liquid medium. Briefly, 1.5 ml of culture was centrifuged at 6000 rpm for 5 minutes at 4°C and supernatant was removed. The pellet was then suspended in 0.3 ml of 30 mM distilled water then washed by centrifugation at 6000 rpm for 3 minutes. The pellet was then suspended in 0.3 ml TE buffer, 30 µL of 10% SDS and 1 µL of 20 mg/ml proteinase K, and was incubated at 37 C for 1 hr. After that, 60 µL of 5M NaCl and 50 µL of CTAB (10%) were added and the mixture was incubated at 65°C for 20 minutes and then cooled to room temperature. Then phenol extraction was done by adding 440 µl of phenol-chloroform-isoamyl alcohol (25:24:1) to the mixture above and vortexed, centrifugation at 15000 rpm for 10 minutes at room temperature. The aqueous phase was moved to a new tube and mixed with 440 µl of the phenol-containing solution described above, vortexed and 200 µL TE buffer was added. Centrifugation at 15000 rpm was done for 10 minutes at room temperature and the aqueous solution was mixed with 440 µL of cold isopropanol plus 30 µL of 3 M sodium acetate. Centrifugation was again done at 15000 rpm for 10 minutes at 4°C and the supernatant discarded. The resultant pellet was air-dried and dissolved in 200 µL TE buffer, then 20 µL of 10 mg/ml RNAse were added and incubated at 37°C for 1 hour. After incubation, DNA was extracted with 200 µL of phenol-containing solution, and supernatant was discarded followed by addition of 600 µL of 100% ethanol plus 20 µL of 3 M sodium acetate, and was centrifuged at 15000 rpm for 10 minutes at 4°C. The supernatant was discarded and 150 µL of 70% ethanol was added to wash. The DNA was centrifuged at 15000 rpm for 3 minutes, and the pellet was air dried and was dissolved in 100 µL of TE buffer. Gel electrophoresis was then done to confirm the quality and quantity of extracted DNA.

3. Plasmid rescue from transconjugants

Briefly, the extracted *Rs*1002 genomic DNA from transconjugants was digested by *Hin*dIII. Genomic DNA from *Pto* DC3000 was digested by *spe1* and *Mlu1*. This was then followed by ethanol precipitation of the DNA and then air drying for 10 minutes. After drying of the DNA pellet, 2.5 µL water was added then vortexed before addition of 2.5 µL of 2 × ligation mixture and kept at room temperature for 30 minutes. Then in ice, this ligation mixture was transformed into 50 µL of competent cell (DH5α), and then heat-treated at 42°C for 50 seconds. This was

followed by the addition of 500 ml LB liquid medium and incubated at 37 °C in an incubator with shaking for 30 minutes. The mixture was centrifuged at 4000 rpm for 3 minutes, 300 µL of top aqueous solution was discarded. Selection was then done by spreading of 150 µL of this mixture on LB agar plate containing kanamycin and incubation at 37°C overnight. Colonies that grew on kanamycin were replica plated on selection plate containing LB kanamycin.

4. Plasmid DNA extraction

Plasmid DNA was extracted from some colonies that grew on LB Km plate. First, using a tooth pick a colony was picked and grown in 3 ml LB liquid medium overnight. Then 1 ml of the culture was pippeted into eppendorf tube and centrifuged at 5000 rpm for 5 minutes. The supernatant was then discarded, and final centrifugation was done for 1 minute to remove all the supernatant. To the pellet, 100 µl of resuspension buffer was added, vortexed and placed on ice for 5 minutes followed by addition of 200 µL of lysis buffer mixed well by gently turning the tubes up and down and placed in ice for 5 minutes. To this solution, 150 µl of neutralization buffer was added then kept in ice for 10 minutes followed by centrifugation at 15000 rpm for 10 minutes at 4°C. Next, phenol extraction was done followed by ethanol precipitation of the DNA and washing using 150 µL of 70% ethanol. The DNA pellet was dissolved in 200 µL TE buffer by vortexing, before addition of 1 µL of RNAse, mixed briefly and spun down and incubated at 37°C for 30 minutes then briefly cool down. To this solution, a second phenol extraction was done followed by ethanol precipitation and washing with 100 µL 70% ethanol mixed and centrifuged at 15000 rpm and supernatant discarded. The pellet was then dried and dissolved in 100 µL distilled water. Quality of DNA was confirmed by 1.4% gel electrophoresis.

5. Sequencing of rescued plasmids

The plasmid DNA obtained was then utilized as template for BigDye terminator sequencing using M13F primer.

6. Sequence investigation

Sequences obtained from flanking regions of the transpson were mapped on to the genome of *R. solanacearum*; https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi. By using the

BLASTP algorithm, the obtained sequences were matched to those protein sequences in the NCBI for homology.

7. Inactivation of ralhibitins with insensitive bacteria

The mechanism for specific growth inhibition of RS1002 is not clear yet. Most bacteria tested are insensitive to ralhibitins or they can inactivate or detoxify them. I examined whether ralhibitins cultured with different bacteria such as *Bulkhoderia glumae* (*Bg*)*, Pectobacterium carotovorum* subsp*. carotovorum* (*Pcc*) and *Rhizobacteria radiobacter* (*Rr*) still retained the capacity to inhibit growth of RS1002. Briefly, *R. solanacearum*, *Bg, Pcc* and *Rr* were obtained from glycerol stocks stored at -80°C and streaked on BG, YP, KB and LB agar plates, respectively and incubated at 27°C overnight. Then a colony from each bacterium was grown in 3 ml liquid medium at 27°C shaking at 200 rpm. From the overnight culture, 10 ul was obtained and dropped into 3 ml fresh medium for each bacterium and briefly mixed. Further, 3 µL of each bacterial suspension was added to fresh 3 ml liquid medium plus 3 µL of ralhibitins A-E making a final concentration of 10 µg/ml. Control tubes had respective bacteria without addition of ralhibitin. This was incubated overnight at 27°C shaking at 200 rpm. To obtain spent culture from the overnight bacterial-ralhibitin mixture, filtration was done using 0.22 µm filters. The resultant suspension (spent culture) was then transferred to new tubes and then 70 µL of fresh *R. solanaceaurum* overnight culture was added and incubated overnight at 200 rpm with shaking. Bacteria absorbance was then measured.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

I. CONCLUSIONS

1. Identification of compounds (ralhibitins) inhibiting *R. solanacearum*

To identify chemical compounds that inhibit phytopathogenic bacteria is a critical goal in production of crops. The second most important bacterial plant pathogen is bacterial wilt caused by *Ralstonia solanacearum* and affects over 400 crop species globally (Mansfield et al., 2012). Control options against bacterial pathogens such as *R. solanacearum* are limited. The use of certified seed, field sanitation, sterilization of tools and rotation of crops have not been effective in the management of the pathogen. Similarly, conventional pesticide application has not solved this problem due to low efficacy and negative effects to the environment. This calls for increased search for better, effective and economically viable alternative bactericides. Screening for compounds that can control *R. solanacearum* from a pilot chemical library identified one compound 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline that could inhibit its growth.

2. Identification of functional active group

Structure activity relationship studies helped unravel the active functional group in the effective compound 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline against *R. solanacearum*. The presence of a halogen group at the para position of the benzene ring enhanced the activity of this compound. The presence or lack of methoxy group did not affect the activity of the effective compound. However, the introduction of a different functional group such as hydroxyl or methyl to the para position of the effective compound abolished its activity. However, even with halogens introduction, their position was important because when chlorine was introduced at the meta position of the benzene ring, activity was also abolished. Similar abolishment of activity was also accomplished by the introduction of an extra benzene ring. Therefore, the most important discovery from structure activity relationships is that the active functional group of the effective compound was the presence of a halogen at the para position of the benzene ring.

3. Efficacy of analogues against *R. solanacearum*

The discovery of the active functional group in the identified effective compound, 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline (ralhibitin A) helped synthesize new analogues (B-F). The analogues replaced the bromine at the para position in the effective

compound with chlorine, fluorine or iodine. Also, the new analogues were with or without the methoxy group. Comparison of the activity efficacies of effective ralhibitin A and its analogues (B-F) against *R. solanacearum* revealed that the most active compounds were A, C and E. Both ralhibtin A and C had a bromine at the para position of the benzene ring with the only difference being with or without a methoxy group whereas ralhibitin E had an iodine group at the para position of the benzene ring. The second most active compound against *R. solanacearum* was ralhibitin B and lastly ralhibitin D. Ralhibitn B and D had a chlorine at the para position of the benzene ring differing only by the presence or lack of a methoxy group. However, ralhibitin F containing fluorine at the para position of the benzene ring was found to have poor activity against *R. solanacearum*. Therefore, from this analysis, structure activity relationship is important understanding the active component resulting in development of better derivatives.

4. Properties of ralhibitins

Ralhibitins were determined to be highly thermostable and could also tolerate a range of pH. Ralhibitin E was determined to have a significant time dependent killing effect on *R. solanacearum*. Additionally, *R. solanacearum* could grow in spent culture medium of ralhibitin insensitive bacteria implying that these bacteria could detoxify or inactivate their inhibitory effect.

5. Species-specific activity of ralhibitins

Ralhibitins effective against *Ralstonia solanacearum* were evaluated against other Gram-positive and Gram-negative bacterial pathogens. Ralhibitin A-E inhibited the growth of *Ralstonia solanacearum* strains and *Xanthomonas oryzae* pv. *oryzae* strains. Ralhibitin E containing an iodine group besides inhibiting *Ralstonia solanacearum* and *Xanthomonas oryzae* pv. *oryzae* also completely inhibited the growth of *Clavibacter michiganensis* subsp. *michiganensis* and *Xanthomonas campestris* pv. *campestris* at 10 µg/ml final concentration. Dose dependent assays revealed that the most effective Ralhibitin against *Xanthomonas oryzae* pv. *oryzae* was D at MIC of 5 ug/ml.

6. *In planta* protective effect of ralhibtins

Ralhibitin D was able to protect *R. solanacearum* infected tomato seedlings in a dose dependent manner under low and high bacterial inoculum. Protective ability was observed at low and high

pathogen inoculum levels. The protective effect was achieved at a chemical concentration of 56 µg/ml.

7. Mode of action of ralhibitins

Efforts to understand the mode of action of ralhibitins through the use of random transposon mutagenesis were unsuccessful. Therefore, the target site of the ralhibitins remains unraveled.

II. RECOMMENDATIONS

Future work should focus on finding out the mechanism of ralhibitins to inhibit susceptible bacteria. Additionally, the mechanism of tolerance to ralhibitins in many tested bacteria should also be unraveled. Having determined that the ralhibitins inhibit the growth of other bacteria pathogens such as *Xoo, Xcc* and *Cmm* in vitro, *in planta* studies should be done to determine their protective effects on rice, brassicas and tomato plants respectively. Finally, studies to determine the protective effect of ralhibitins in the naturally infected fields should be done to enable their commercial exploitation for the benefit of farmers.

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